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STIC-ILL

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EXPRESSION OF A CHLAMYDIA-TRACHOMATIS MAJOR OUTER MEMBRANE PROTEIN SEROTYPE SPECIFIC EPITOPE AS A LAM-B HYBRID PROTEIN IN AVIRULENT SALMONELLA-TYPHIMURIUM
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ABSTRACTS

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E-5 Colonizing ability and immunogenicity of a galE mutant of an enteroadherent Escherichia coli. A. CRAVIOTO*, R.E. REYES, O. BENITEZ, A. TELLO, F. TRUJILLO AND V. VAZQUEZ. Instituto Nacional de Ciencias y Tecnología-DIF, Mexico City.

A spontaneous mutant of an enteropathogenic Escherichia coli (EPEC) of serotype O111:H2 carrying an 0.5 kb deletion in the galE region of its chromosome, capable of expressing a 94 kDa plasmid-coded outer-membrane-protein (OMP), which has been associated with enteroadherent ability of EPEC was tested for safety and immunogenicity in adult volunteers after written informed consent was obtained. Twenty-two volunteers were divided at random into two groups in a double blind study and inoculated orally with 6×10^{10} bacteria/ml of the galE mutant and of a non-pathogenic fecal E. coli. None developed diarrhea after inoculation. Fecal samples were obtained daily for 10 days and tested for the presence of galE mutants of E. coli after growth in nutrient agar containing 5.5 mM D-galactose and 0.005% neutral red. Growth in these plates was transfered to Whatman 541 filters and tested by DNA hybridization for the EAF plasmid which codes for the 94 kDa OMP. Mutant strains were isolated from all vaccinated subjects 48 hours after inoculation; these strains became the predominant aerobic flora over the next 5-6 days, disappearing from the feces by day 10 post-inoculation. A four-fold or greater rise in serum antibody titers to the O111 LPS and to the purified 94 kDa OMP was observed in 92% of vaccinated individuals.

E-6 Genetic Fusion of a Peptide From Streptococcus mutans Glucosyltransferase B to Cholera Toxin B Subunit: Structure and Immunogenicity. M. DERTZBAUGH* and F. MACRINA. Virginia Commonwealth University, Richmond, VA 23298-0678.

The B subunit of cholera toxin (ctxB) has been reported to elicit immunity to heterologous protein antigens when co-presented to the intestinal mucosa. Most proteins are poor immunogens when administered orally, and the use of ctxB may aid in the development of oral subunit vaccines. We are exploring this possibility by constructing a ctxB fusion protein using a portion of the glucosyltransferase B enzyme (gtfB) from the cariogenic bacterium Streptococcus mutans. A synthetic oligonucleotide corresponding to the amino acid sequence of an antigenic region of gtfB was inserted upstream of the ctxB gene and transformed into E. coli. The chimeric protein retained its ability to bind GM_1 ganglioside, which is a property of ctxB. However, gel filtration chromatography revealed the quaternary structure of the chimera to be a trimer, instead of the pentamer normally formed by ctxB. Both secretory IgA and serum IgG antibodies were elicited to the gtfB and ctxB gene products upon feeding 500 μg of the chimera to mice. The results demonstrate the immunogenicity of the chimera and provide us with a model for evaluating the efficacy of ctxB chimeras as oral vaccines.

E-7 Adjuvant Effect of B-Subunit of E. Coli Heat Labile Toxin on Secretory Antibody Response to Oral Influenza Virus Immunization in Mice. K.-C. BERGMANN, R.H. WALDMAN*, M. BICAK, and G. J. RUSSELL-JONES. The Karl-Hansen-Klinik, Bad Lippspringe, FRG, University of Nebraska Medical Center, Omaha, Nebraska, and Biotechnology, Australia, Roseville, NSW.

Oral immunization has been shown to be a convenient and effective method of stimulating secretory antibody (SAb) at distant mucosal surfaces such as the respiratory tract. A disadvantage of oral immunization is the large amount of antigen necessary. Other studies have suggested that the non-toxic B subunit of E. Coli heat labile toxin (LTB) might function to enhance the immune response to orally administered antigens. The purpose of this study was to determine if mice receiving an oral inactivated influenza virus vaccine would have an enhanced immune response if the vaccine were mixed with LTB. 70 CF-1 strain mice were divided into 5 groups: (a) lower dose (0.25 ml vaccine/dose), (b) higher dose (0.50 ml), (c) lower dose + LTB (0.25 mg), (d) saline control, and (e) LTB control. Results show that oral immunization stimulates a SAb response which is primarily IgA, and that the magnitude of the response is affected by the dose of vaccine. There was approximately a 7-fold greater respiratory tract IgA antibody response in mice given the vaccine plus adjuvant, and more than a 4-fold greater respiratory IgG antibody response, as compared to mice receiving the vaccine alone. Even when compared to mice receiving a higher dose of vaccine (twice the amount of HA), the mice receiving lower dose plus adjuvant showed a significantly greater antibody response (nearly 3-fold greater for IgA, 4-fold greater for IgG).

E-8 Expression of a *Chlamydia trachomatis* Major Outer Membrane Protein Serotype-Specific Epitope as a LamB Hybrid Protein in Avirulent *Salmonella typhimurium*. Y.-X. ZHANG*, H. SU, Y.-Q. ZHU, and H. D. CALDWELL, LMSF, Rocky Mountain Labs, NIAID, NIH, Hamilton, MT 59840.

Stimulation of mucosal immune responses specific to *C. trachomatis* serotype-specific antigenic determinants is thought to be a necessary requirement for the development of a vaccine for blinding trachoma and perhaps sexually transmitted infections caused by chlamydiae. A possible approach for evoking chlamydial serotype-specific mucosal immunity is through the use of infectious enteric vaccine vectors that express this antigen or its immunologically relevant determinants at their cell surface. The chlamydial major outer membrane protein (MOMP) is the location of serotype-specific determinants. For serotype A, type-specific determinants map to variable domain I of the protein and reside within a contiguous ten amino acid sequence (residues 65-74) of this domain. We have genetically fused this type-specific amino acid sequence and a trimer of the sequence into the LamB protein using the plasmid vector pJAC-264 and stably expressed the hybrid molecule in the outer membrane of the *aroA* vaccine strain of *S. typhimurium*. The expression of the serotype-specific determinant as a LamB antigenic hybrid molecule was demonstrated by western blotting with a monoclonal antibody specific for the inserted sequence and by direct sequencing of the recombinant plasmids.

E-9 Virulence and Vaccine Potential of phoP Mutants of Salmonella typhimurium. J. E. Galan*. Washington University, St. Louis, MO.63130.

We have constructed phoP mutants of S. typhimurium strains SR-11 and SL1344 and investigated their virulence and vaccine potential. Balb/c mice survived challenge with a number of organisms that represent 10^4 to 10^5 times the per oral and intraperitoneal LD_{50} dose of the wild-type parent strains. phoP mutants established an infection of Peyer's patches for up to 7 days after inoculation and induced a delayed (footpad) hipersensitivity reaction against Salmonella antigens, an indication of their ability to stimulate a cell mediated immune response. Immunized mice resisted challenge with 10^3 to 10^4 LD_{50}'s of wild type S. typhimurium SR-11. These results indicate that phoP mutants of S. typhimurium represent candidate vaccine strains and potential carriers to deliver heterologous antigens to the gut-associated lymphoid tissue (GALT).

E-10 Salmonella choleraesuis mutants lacking genes for adenylate cyclase and the cAMP receptor protein are avirulent and immunogenic. SANDRA M. KELLY*, BETH A. BOSECKER and ROY CURTISS III, Washington University, St. Louis, Missouri.

Salmonella typhimurium Δcya and Δcrp mutants have been found to be avirulent and immunogenic in BALB/c mice (Curtiss and Kelly, 1987. Infect. Immun. 55:3035). Although galE and ΔaroA mutations render S. typhimurium both avirulent and immunogenic, galE S. choleraesuis mutants are neither avirulent or immunogenic whereas ΔaroA mutants are avirulent but not immunogenic when administered intraperitoneally to mice (Nnalue and Stocker, 1986. Infect. Immun. 54:635; 1987. Infect. Immun. 55:955). S. choleraesuis mutants with crp::Tn10, cya::Tn10, and fusaric acid resistant derivatives with deletions (Δ) of the Tn10 and adjacent DNA sequences were constructed. These Tn10-insertion mutants and their Δcya and Δcrp derivatives were evaluated for virulence in 8-week old BALB/c mice. Mice survived oral inoculation with crp::Tn10, cya::Tn10, Δcya and Δcrp strains at doses equivalent to 3×10^3 times the LD_{50} dose for the wild-type S. choleraesuis parent. Thirty days after immunization, mice were protected against oral challenge with 3×10^3 times the LD_{50} dose of wild-type S. choleraesuis. The crp::Tn10 and Δcrp mutants were slightly more immunogenic than the cya::Tn10 and Δcya mutants.

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Oral immunization against chlamydial eye infection.
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Oral Immunization Against Chlamydial Eye Infection

Hugh R. Taylor, Elaine Young, A. Bruce MacDonald,* Julius Schachter,† and Robert A. Prendergast

The effects of enteric administration of different preparations of *Chlamydia trachomatis* prior to ocular challenge with live chlamydia were compared to the immunity that develops after recovery from ocular infection. Oral immunization with either live homologous serovar B or with formalin-killed heterologus serovar L_2 did not influence the response to subsequent ocular challenge. Although oral immunization with live serovar led to protection against heterologous ocular challenge with serovar B, oral immunization with noninfectious UV-irradiated serovar L_2 led to more severe and prolonged disease. An immunizing regimen designed for maximal mucosal and systemic immunity also resulted in protection against homologous ocular challenge. Although protection was correlated with the presence of serum IgA antibodies, no clear mechanism for the protective ocular immunity to chlamydial infection has emerged. These studies show that it is possible to stimulate mucosal immunity to induce protection against subsequent ocular challenge with *C. trachomatis* that is equal to that which follows prior ocular infection. Invest Ophthalmol Vis Sci 28:249–258, 1987

In an animal model for trachoma, monkeys develop resistance to chlamydial eye infection after recovering from a primary ocular infection.[1] However, this resistance is only partial, since animals still develop infection following challenge, although it is milder and of shorter duration.[2] Although there is evidence for protective immunity in chlamydial infection, much of the tissue damage associated with trachoma also appears to be immunologically mediated.[3] It has been suggested that the antigens responsible for protection may be different from those causing the deleterious reactions[4]; if it were possible to specifically stimulate only the protective immune response, then this might result in a safe and effective trachoma vaccine. Such a vaccine obviously would have a tremendous impact on the many millions of children who live within endemic areas.

The successful cultivation of *Chlamydia trachomatis* in the early 1950s opened the way for attempts to develop vaccines against trachoma. During the 1960s, a number of groups carried out studies on trachoma vaccines in animals and in humans with vaccines designed to stimulate systemic immunity.[4,5] These preparations contained, almost without exception, killed whole elementary bodies (EB) and were given systemically either by intramuscular or subcutaneous injection.[6–8] The overall experience of these studies was that some mild, limited protection often did occur, but it was, at best, of short duration, lasting less than 2 yr in humans.[4,5,9] Furthermore, several of these studies also showed that a worsening of disease, or hypersensitivity to chlamydia, could be induced by an "inadequate" vaccination with either low potency vaccines or challenge with heterologous serovars.[4] Because of both the lack of efficacy and concerns about safety, interest in trachoma vaccines waned.

In humans, infection with *C. trachomatis*, with the exception of the invasive L serovars, appears to be almost always confined to the mucosal surface.[10] Many of the complexities of the mucosal immune system have been elucidated in the last few years. For example, successful regimens for specifically stimulating mucosal immunity have been successfully exploited for other infections such as cholera,[11] where preceding systemic vaccines were of only limited efficacy. Previous studies in monkeys and rats have shown that enteric presentation of antigen leads to antigen-specific priming of the conjunctiva,[12,13] and preliminary studies in guinea pigs suggest partial protection to ocular challenge with *C. psittaci* may follow distant mucosal infection.[14] In addition, there have been tremendous advances in the methods for identifying and producing purified antigens in large amounts,[15,16] should an appropriate antigen be identified.

For these reasons, it seemed appropriate to reexamine the possibility of developing a vaccine for trachoma that specifically stimulates mucosal immunity, is easy to administer, and does not induce hypersen-

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sitivity. This report presents a series of studies of the effect of distant mucosal immunization with purified chlamydial EB on subsequent ocular infection with *C. trachomatis* in a monkey model.

Materials and Methods

Animals

Groups of young adult cynomolgus monkeys were obtained from Hazelton Laboratories (Alice, TX). There were five monkeys in each group. All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research.

Preparation and Administration of *C. trachomatis*

Serovar B (HAR-36) and L_2 were mass-cultured in tissue culture. Purified EB were prepared by centrifugation through renograffin[15] and resuspended at the appropriate dilutions in phosphate-buffered saline. *C. trachomatis*, serovar E (Bour strain), was grown in the yolk sac of embryonated hen eggs, and EB were purified by centrifugation through renograffin.

Ocular inoculations were adjusted to 1×10^5 infection-forming units (IFU) per ml, which is equivalent to $10^{3.2}$ egg lethal dose 50 per ml. Twenty microliters of suspension was placed into each conjunctival sac, giving an ocular inoculation of approximately 2×10^3 IFU per eye.

Enteric doses consisted of 5×10^8 EB. Monkeys were fasted over night; immediately after nuetralizing gastric contents with 5 ml of sodium bicarbonate solution, the enteric dose was administered via a gastric tube.[13] Rectal inocula contained 1×10^5 IFU and was administered directly in the rectum during proctoscopy. Preparations for intramuscular injection contained 5×10^{10} EB per ml. This had been emulsified 1:1 with complete Freund's adjuvant for the initial injection and with incomplete Freund's adjuvant for subsequent injections. At each time, animals were given a total of 1 ml at two sites.

Where indicated, viable agent was inactivated either by ultraviolet irradiation (UV) or by the addition of formalin. For UV inactivation, a suspension of EB was placed in a plastic dish to a depth of 2 mm and the open dish placed 10 cm from a Westinghouse 40 W germicidal UV lamp (George W. Gates and Co., Inc., Franklin Square, NY) for 120 min. The suspension was continuously agitated by magnetic stirrer. Alternatively, EB were inactivated by the addition of formalin to a final concentration of 0.01%. Inactivation was confirmed by three serial culture passages using multiple dilutions of the inactivated agent. The detailed immunizing protocol and ocular challenge for each group of monkeys are given in the results section and in the respective figures.

Examination and Specimen Collection

The clinical response of each eye was graded for a number of individual signs that were then combined to express the clinical response as two simplified indices as previously described.[1,17] Briefly, the "follicular index" quantitates the follicular response in the bulbar, limbal, superior tarsal, and superior fornix conjunctiva. The "inflammatory index" summarizes the nonspecific signs of inflammation: specifically, hyperemia or injection of the bulbar, superior tarsal, superior fornix conjunctiva, and ocular discharge.[17] Examinations were performed in random order without informing the examiner of the monkey identification. Recovery from disease was defined as a follicular index of less than one and an inflammatory index of zero.

Conjunctival swabs were collected at each examination for chlamydial reisolation cultures in a cycloheximide-McCoy cell tissue culture system.[18] Although both eyes were examined, specimens were taken only from the left eye to eliminate the possibility of artifactitious changes in the right eye. Rectal swabs were also taken for culture from monkeys in one group.

Serology

Tears and serum were collected for microimmunofluorescent serologic tests against whole chlamydial EB.[1,19] Each specimen was separately titered against purified preparations of serovar B and serovar L_2 EB except for specimens from the maximally immunized group, which were assayed against serovar E EB. Commercially available reagents cross-reacting with monkey immunoglobulin heavy chains were used: goat antihuman IgG (Hyland Labs; Costa Mesa, CA) and goat antihuman IgM (Kallestad; Austin, TX). IgA was assayed using rabbit antimonkey IgA (Nordic Immunological Labs; El Torro, CA).

Lymphocyte Proliferation Assay

Peripheral blood mononuclear leukocytes (PBML) were isolated from animals as previously described.[20] PBML were suspended at a concentration of 1.5×10^5/ml in Minimum Essential Medium (MEM) supplemented with 25 mM Hepes buffer, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (all from Gibco; Grand Island, NY). Triplicate wells containing 1.5×10^5 PBML and 100 μl of antigen (10^7 serovar L_2 EB) or medium in a total volume of 200 μl were cultured in round bottom plates (Linbro; McLean, VA) at 37°C in 5% CO_2. One μCi ^{3}H-thymidine was added to each well on day 3, cultures were harvested 18 hr later, and radioactivity was measured in a liquid scintillation counter. The Stimulation Index (SI) was determined as the ratio of

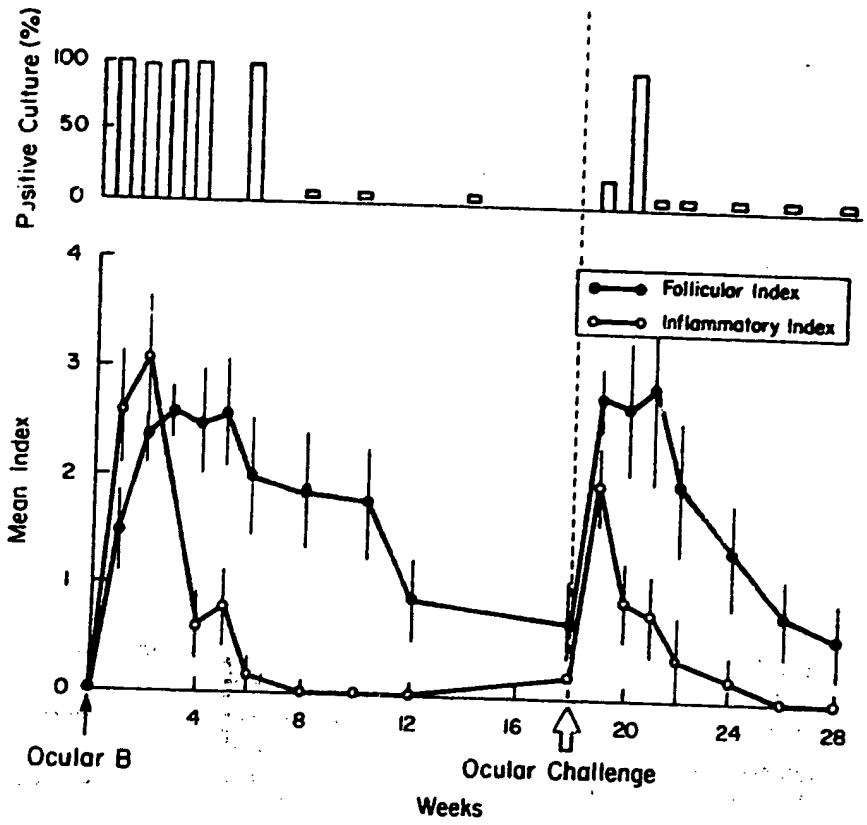

Fig. 1. Clinical ocular response of five naive monkeys given a single ocular inoculation of live serovar B *C. trachomatis* on week 0 and challenged with the same agent on week 18. The mean follicular and inflammatory indices (*bottom*) and the frequency of positive chlamydial cultures (*top*) are shown. Error bars represent standard error of the mean.

counts per min (cpm) in test wells over cpm in contr[illegible] wells.

Results

Primary and Secondary Responses in Nonimmunized Monkeys

Five naive monkeys receiving a single ocular inoculation with live *C. trachomatis*, serovar B, rapidly developed an acute, self-limited inclusion conjunctivitis (Fig. 1). Nonspecific signs of inflammation (inflammatory index) resolved within 1 month, whereas the follicular response (follicular index) waned more slowly. All animals had clinically recovered by 12 wk. After recovery, these animals demonstrated ocular immunity to chlamydial infection and were relatively resistant to ocular challenge with the same organism (Fig. 1). Clinical disease resolved within 6 wk of challenge. Positive chlamydial cultures isolated from conjunctival swabs were obtained for 6 wk after the initial infection but only for 2 wk after ocular challenge.

These monkeys had a strong, serovar-specific antibody response with IgM, IgG, and IgA antibodies appearing in both serum and tears following primary infection (Fig. 2). Serum serovar-specific IgG titers increased with secondary challenge with little change in the titers of the other antibody classes.

The Effect of Oral Immunization With Live Agent

Two groups of five monkeys each were immunized orally at weeks 0, 1, and 2 with live chlamydia of either serovar B or serovar L_2 and given an ocular challenge with serovar B at week 3. The clinical and microbiological response of monkeys orally immunized with live serovar B EB was essentially the same as nonimmunized animals (Fig. 3A). They had recovered clinically within 12 wk, and positive chlamydial cultures persisted for 4 wk. However, monkeys immunized with live serovar L_2 had milder disease than nonimmunized monkeys (Fig. 3B) and responded in a similar way to ocular immune animals (vide supra). Clinical disease resolved in 7 wk, although one animal had mild persistent clinical disease and gave weakly positive conjunctival cultures for 8 wk. One monkey fed live serovar L_2 died 5 wk after ocular challenge. No evidence for disseminated chlamydial infection was found on autopsy.

Monkeys immunized with live serovar B developed moderate serum serovar B-specific IgM and IgG titers within 2 wk of immunization, but neither serum IgA antibodies nor tear antibodies of any class were detected until after ocular challenge (Fig. 4A). Two weeks after ocular challenge, there was a rapid appearance of antibodies of all three isotypes in the tears. There was a

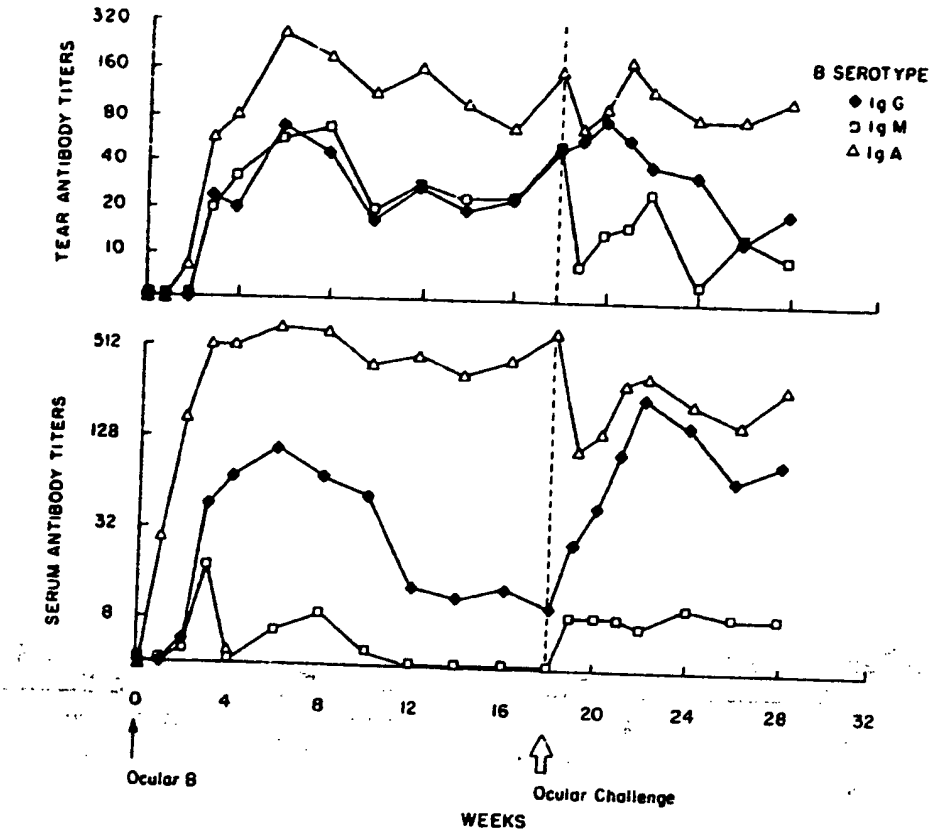

Fig. 2. Mean serovar B-specific antibody titers in tears (*top*) and serum (*bottom*) of five naive monkeys given a single ocular inoculation of live serovar B *C. trachomatis* on week 0 and challenged with the same serovar on week 18

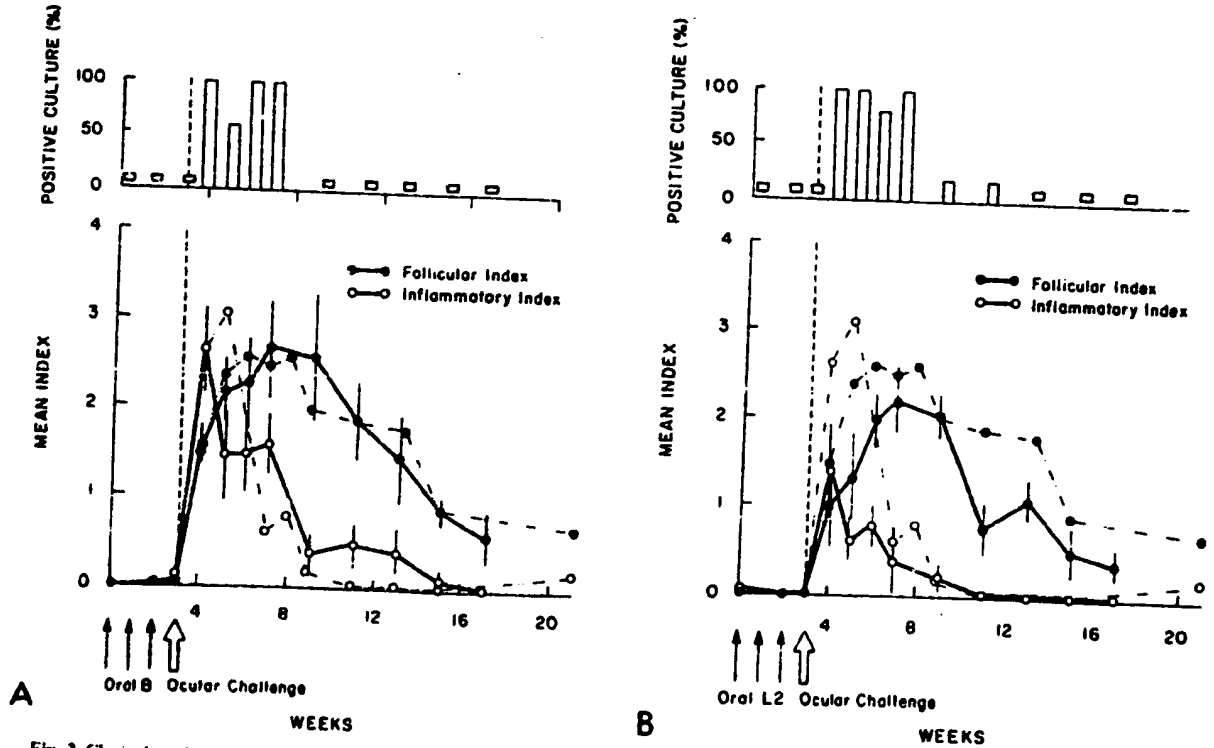

Fig. 3. Clinical ocular response following challenge with serovar B of (A) five monkeys orally immunized with viable serovar B *C. trachomatis*; (B) five monkeys orally immunized with viable serovar L_2 *C. trachomatis*. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. The response of naive animals is shown in broken lines for comparison. Error bars represent SEM.

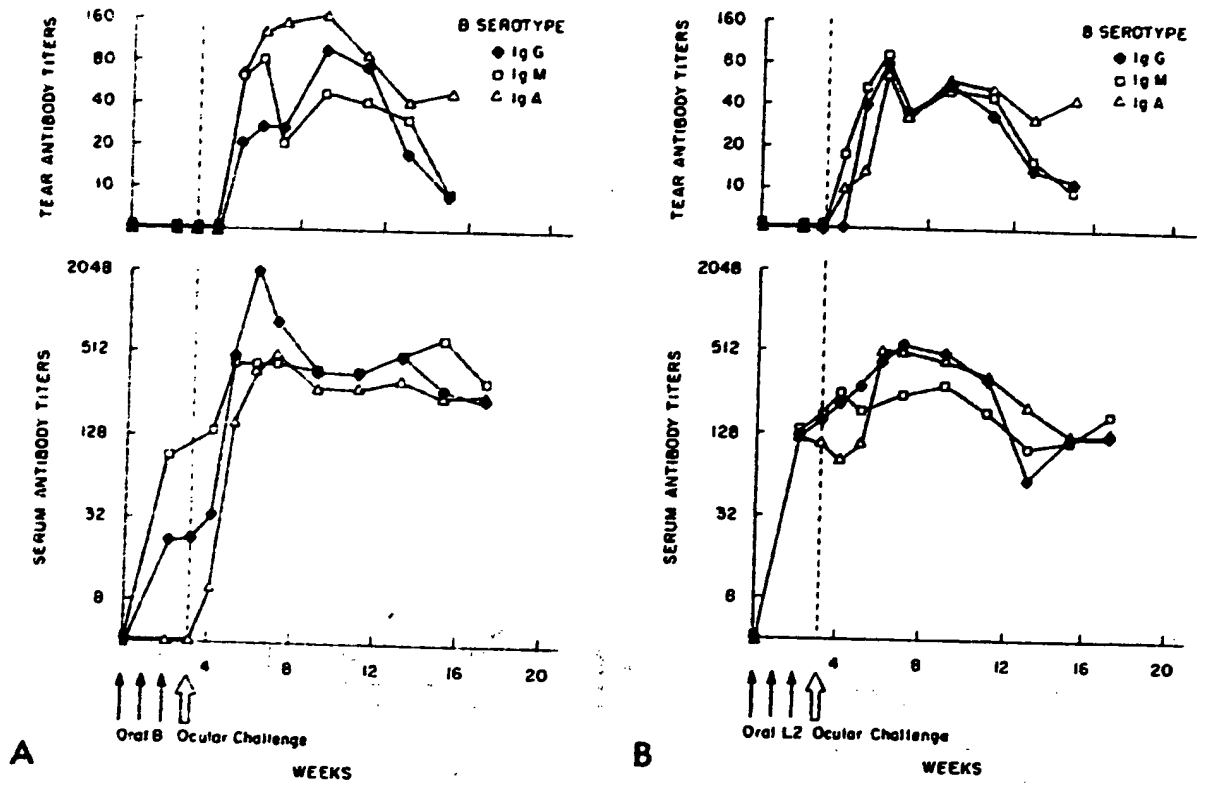

Fig. 4. (A) Mean serovar B-specific antibody titers in tears and serum of five monkeys orally immunized with viable serovar B *C. trachomatis* and challenged with the same serovar at week 3; (B) five monkeys orally immunized with viable serovar L_2 *C. trachomatis* and challenged with serovar B at week 3.

corresponding increase in serum IgM and IgG, and IgA also appeared in serum at this time. The monkeys immunized with live serovar L_2 developed comparable serum serovar B-specific IgM and IgG titers but also developed serum IgA antibodies after immunization (Fig. 4B). Antibodies were not detected in tears before challenge, although a rapid tear antibody response followed ocular challenge. In this group, IgA and IgM could be detected within 1 wk of challenge compared with 2 wk in the animals orally immunized with serovar B and 3 wk in nonimmunized animals. High titers of IgM persisted in the serum and tears of both immunized groups of monkeys. For both immunized groups, the mean serovar-specific antibody titers for serovar B or serovar L_2 for each antibody class did not differ by more than one dilution (data for serovar L_2 not shown).

The Effect of Oral Immunization With Inactivated Agent

Because oral immunization with viable serovar L_2 produced some protection to ocular challenge, we next examined the effect of oral immunization using inactivated serovar L_2. Two groups of five monkeys each were immunized orally following the previous schedule. At weeks 0, 1, and 2, they were given serovar L_2 EB inactivated by either formalin or UV irradiation. They were challenged with ocular serovar B at week 3. The clinical and microbiologic response in the monkeys immunized with formalin-inactivated serovar L_2 EB was similar to that of nonimmunized animals (Fig. 5A). Clinical recovery was seen by 10 wk, although positive conjunctival cultures were obtained for up to 10 wk. In contrast, oral immunization with UV-inactivated serovar L_2 resulted in the marked prolongation of clinical disease and positive culture isolates (Fig. 5B). In this group, disease resolution was delayed for over 5 months, and positive cultures were obtained for up to 10 wk after challenge in 80% (4 of 5) of the animals.

Two monkeys orally immunized with formalin-inactivated serovar L_2 EB developed serovar B-specific IgM antibodies before ocular challenge. These two animals also had low serum titers of antiserovar L_2 IgM and IgG antibodies (titers of 1:16 for each) 3 wk after the initial feeding. After challenge, the serum antibody responses in this group to serovar B (Fig. 6A) and serovar L_2 (data not shown) were essentially the same as nonimmunized monkeys, although lower titers of each

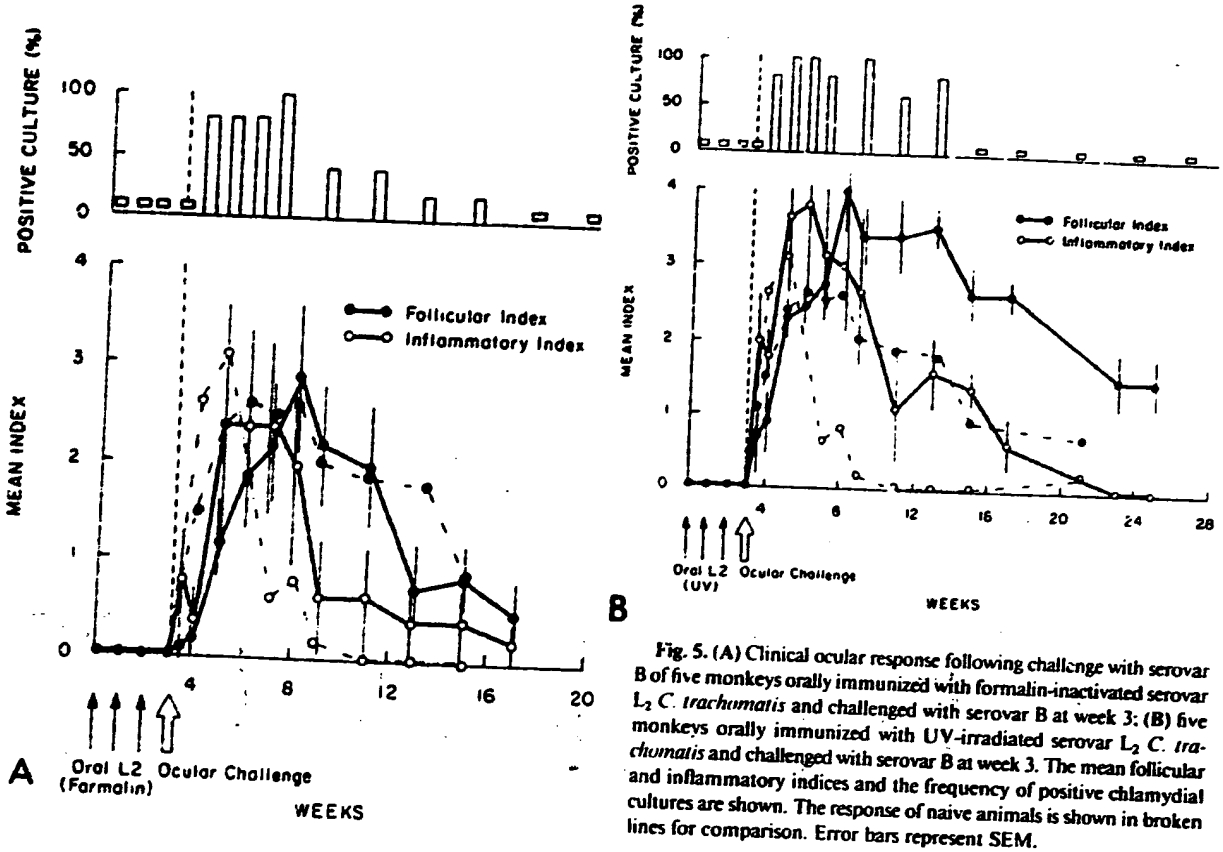

Fig. 5. (A) Clinical ocular response following challenge with serovar B of five monkeys orally immunized with formalin-inactivated serovar L_2 *C. trachomatis* and challenged with serovar B at week 3; (B) five monkeys orally immunized with UV-irradiated serovar L_2 *C. trachomatis* and challenged with serovar B at week 3. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. The response of naive animals is shown in broken lines for comparison. Error bars represent SEM.

isotype were found in tears. Oral immunization with UV-inactivated serovar L_2 EB induced a serum IgM response to both serovar B and L_2 in one monkey (titers of 1:128 for each serovar). Following ocular challenge, the serum antibody response to serovar B (Fig. 6B) was similar to that observed in nonimmunized animals (as shown in Fig. 2), although the IgM response was much greater and more prolonged than in normal monkeys. The tear IgM and IgG response was identical to nonimmunized animals following primary ocular infection; however, the tear IgA response was both delayed and depressed.

The Effect of Maximal Immunization

In an attempt to induce maximal mucosal immunity, six monkeys were given an initial rectal infection on weeks 0 and 5 followed by distant mucosal (oral) and systemic (intramuscular) boosting on weeks 8, 12, and 14. In this experiment, serovar E was used for both immunization and ocular challenge. Mild proctitis developed in all monkeys after the first rectal inoculation with chlamydia, and more marked but still self-limiting proctitis followed the second. Positive chlamydia cultures from the rectum confirmed the establishment of infection (Fig. 7). There was no spread of infection to the eyes following rectal inoculation or oral immunization. After ocular challenge, these animals developed a short-lived but vigorous clinical response (Fig. 7) similar to the ocular immune animals (Fig. 1). Microbiological evidence of infection cleared within 2 wk.

After the first rectal inoculation, serovar E-specific IgM or IgG was not found in serum or tears, but one animal developed low serum titers of IgA (1:32). Following the second rectal inoculation, low titers of serovar E-specific IgG and IgM were found in serum, and moderate titers of IgA were found in all animals (Fig. 8). After oral and systemic boosting, extremely high titers of IgA and IgG and moderate titers of IgM appeared in the serum of all animals. Low titers of IgA and IgG, but not IgM, also appeared in tears at this time. Moderate titers of IgG antibodies (mean titer 1:320) were also detected in rectal secretions, but IgM was not found. Rectal secretions were not tested for IgA. Following ocular challenge, serum IgG and IgM titers did not change, but serum IgA antibodies reached even higher titers (range 28 days post-challenge was 1:16,384 to 1:32,768). Tear serovar E-specific IgA and IgG rose rapidly to very high titers, and low titers of IgM also appeared. The titers of IgG in tears exceeded those in serum, suggesting that local production rather than transudation was responsible for much of the increase in tear antibody.

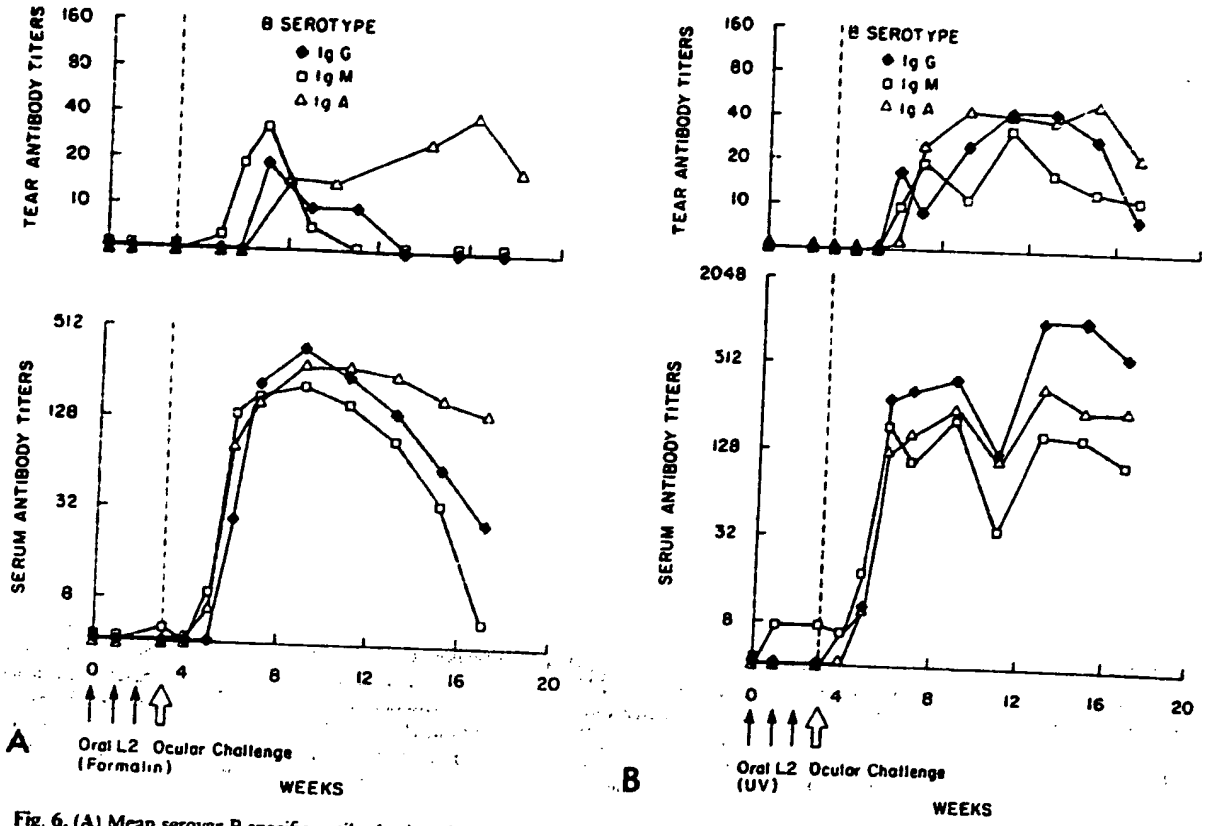

Fig. 6. (A) Mean serovar B-specific antibody titers in tears and serum of five monkeys orally immunized with formalin-inactivated serovar L_2 *C. trachomatis* and challenged with serovar B at week 3; (B) five monkeys orally immunized with UV-irradiated serovar L_2 *C. trachomatis* and challenged with serovar B at week 3.

Cell-Mediated Immunity

Cell-mediated immunity assayed by in vitro lymphoproliferative assays of circulating lymphocytes did not reflect the development of ocular immunity to chlamydial infection. Neither the ocular immune monkeys nor the animals successfully immunized with live serovar L_2 developed a significant cell-mediated immune response. Cellular immunity to whole EB was observed only in animals immunized by intramuscular injection.[20] In this group, the mean SI (± standard deviation) at week 0 was 1.5 ± 0.4 and at week 16 (ocular challenge), 11.2 ± 4.8. Parallel in vitro lymphocyte proliferation assays using purified chlamydial lipopolysaccharide antigen[21] also failed to demonstrate statistically significant sensitization of peripheral blood lymphocytes.

Discussion

The present series of experiments were designed to compare the effect of different immunizing regimens with the ocular immunity that develops following recovery from chlamydial ocular infection. Specifically, we wished to determine whether prior distant mucosal exposure to whole chlamydial EB would protect against subsequent ocular challenge. The oral immunizing

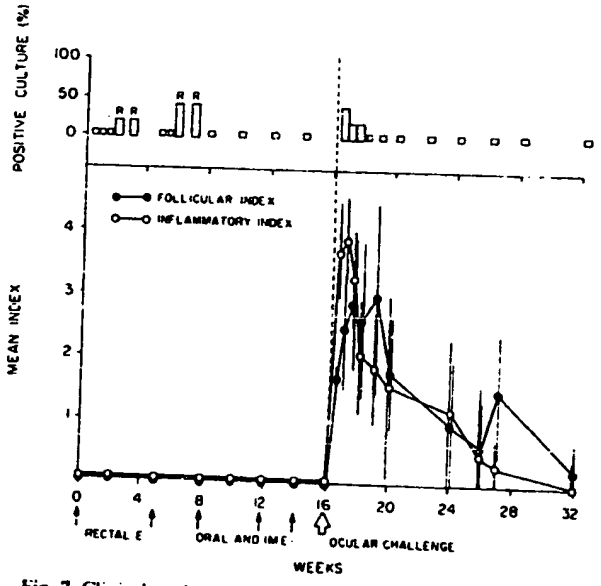

Fig. 7. Clinical ocular response of six monkeys given rectal inoculations of serovar E *C. trachomatis* on weeks 0 and 5; combined oral and intramuscular boosting on weeks 8, 12, and 14; and an ocular challenge with the same serovar at week 16. The mean follicular and inflammatory indices and the frequency of positive chlamydial cultures are shown. For the first 20 wk, both rectal and ocular cultures were taken [(R) indicates positive rectal cultures]. Error bars denote the SEM.

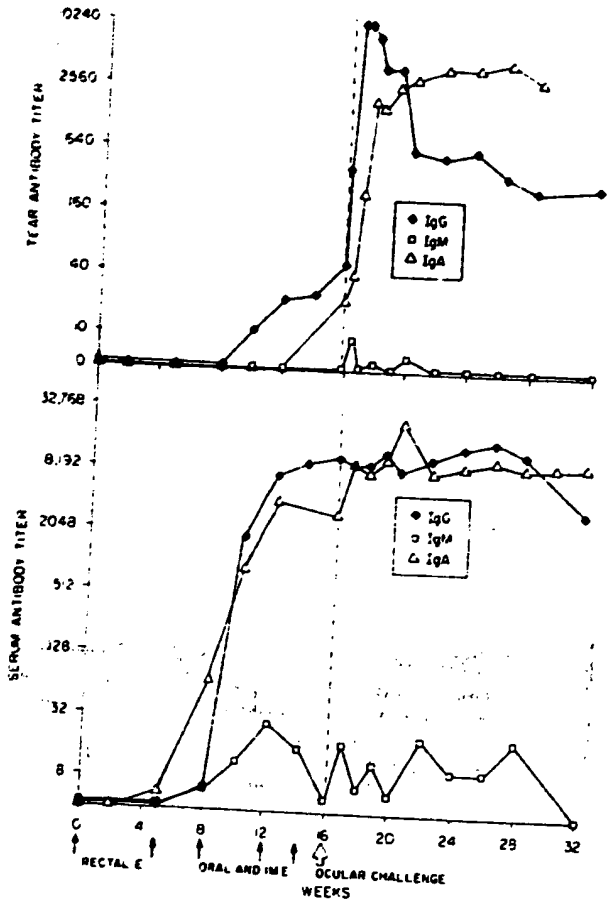

Fig. 8. Serovar E-specific titers in tears and serum of six monkeys given rectal inoculations of serovar E *C. trachomatis* on weeks 0 and 5; combined oral and intramuscular boosting weeks 8, 12, and 14; and an ocular challenge with the same serovar at week 16.

methods were similar to those used in experiments in monkeys with cholera toxoid.[13] Those studies showed that oral immunization would prime the conjunctiva for subsequent ocular challenge. Four chlamydial antigen preparations were investigated for immunization potential: viable homologous EB (serovar B), viable heterologous EB (serovar L_2), and nonviable heterologous EB (serovar L_2) inactivated by either the addition of formalin or UV irradiation. Following oral immunization, resistance to ocular challenge was induced only in animals given viable serovar L_2 EB.

Each of the oral antigen preparations induced detectable serum antibody levels in at least some animals, but serovar-specific antibodies were not found in the tears of any orally immunized animal before ocular challenge. The highest serum antibody titers were in the groups orally immunized with viable organisms. Only the group given viable serovar L_2 had detectable levels of IgA antibodies in serum prior to challenge, and these animals had a brisk tear antibody response after ocular challenge. Both of these features are indicative of priming of mucosal immunity.[22,23] There was no evidence for gastrointestinal colonization by viable serovar L_2 in the one animal autopsied 5 wk after ocular challenge. Although rectal cultures were not performed routinely in these monkeys, positive rectal cultures were not obtained in this group or following enteric boosting in the maximally immunized group when rectal cultures were performed routinely. It is of interest that to be effective oral shigella vaccines must consist of living, although not necessarily invasive, organisms.[24] Nonviable organisms were ineffective, and this was not related to the total antigenic mass. It is believed that the nonviable bacteria are not adequately presented by the antigen-presenting cells in Peyer's patch. Whether similar mechanisms operate for chlamydia is unknown. With viable serovar L_2 organisms, there is the potential for invasion and replication in lymphoid tissue that does not exist for the other serovars, and this could markedly alter the immunizing potential of viable serovar L_2.

The animals orally immunized with viable serovar L_2, however, were not better protected than monkeys that had recovered from ocular infection. For this reason, the almost heroic combined mucosal and systemic immunizing schedule was used to induce maximum immunity without resorting to ocular infection. These monkeys were partially resistant to challenge, but they were still not better protected than the ocular immune animals, despite extraordinary titers of IgA and IgG in both serum and tears and demonstrable cellular immunity.

From these studies, it is not clear whether components of humoral immunity are important in resistance to ocular infection. Tear antibodies were not induced without ocular infection except in the maximally immunized group, but the brisk tear antibody response that followed ocular challenge did suggest some form of immunologic priming of the conjunctiva in some groups. An analogous situation has been found with oral immunizing studies using cholera toxoid[13] where oral immunization primed the conjunctiva but specific antibody-containing cells were not seen prior to ocular challenge. It is always difficult to tell whether antibodies in tears from inflamed eyes are locally produced in the conjunctiva or are part of the inflammatory transudate from serum. A huge increase in the numbers of B lymphocytes in the conjunctiva has been found following chlamydial infection[25]; moreover, as tear antibody titers may exceed serum titers, it seems likely that, in large part, antibodies found in the tears are locally produced. The presence of antichlamydial IgA antibodies in serum did correlate with resistance to ocular infection, but it is unclear whether this is a direct effect of the serum antibodies themselves or whether

the presence of serum IgA is merely an indicator of some other priming of the immune system against subsequent chlamydial infection. None of the oral immunizing schedules induced tear antibodies prior to ocular challenge, although both ocular immune animals and maximally ..nmunized animals had tear IgA antibodies and were resistant to challenge. In several animal models, it has been the presence of local antibodies rather than serum antibodies that has correlated with resistance to chlamydial infection.[26,27] Whether IgA antibodies in either serum or tears act by preventing new infection or by limiting existing infection is unknown.

A marked worsening of disease or hypersensitivity was induced in animals orally immunized with UV-inactivated serovar L_2 EB. Both formalin treatment and UV irradiation block chlamydial replication, but UV inactivation does not prevent specific attachment and phagocytosis of the EB by cells.[28] Monkeys fed UV-inactivated EB showed no characteristics in their serologic or cellular immune response that could explain their prolonged infection and exaggerated response or that would suggest what mechanisms might be involved. Grayston and Wong have emphasized that "inadequate" trachoma vaccines can induce hypersensitivity,[4] especially if they are of a heterologous serovar as was the case in these monkeys. Heterologous immunization, however, did not lead to sensitization with the ineffective formalin-inactivated EB, and viable serovar L_2 gave good protection even against heterologous challenge with serovar B. It should be noted that serovars B and L_2 are antigenically related, both belonging to the "B group".[19] The comparability of the serovar-specific antibody response in our studies reflects this. It may be that heterologous challenge using organisms from the "C group" would induce more severe disease than heterologous challenge within the same group. However, it seems unlikely that ocular disease could be more severe than the prolonged disease seen in the group orally immunized with UV-inactivated serovar L_2 EB and challenged with B. The mechanisms involved in the hypersensitive response are not clear and require further examination, as they may shed light on the deleterious features of the host immune response to chlamydial infection that must be avoided by any prospective vaccine.

In summary, these studies do show that it is possible by oral immunization to induce some degree of protective immunity to subsequent chlamydial ocular infection. Although this supports the notion that mucosal immunity is important in protecting against chlamydial infection, the overall lack of efficacy of the various oral immunization regimens, except for the pathogenic live serovar L_2, and the failure to obtain better protection than that induced by recovery from previous ocular infection were disappointing. One explanation for this may lie in the use of whole EB as the immunizing antigen. It is now clear that chlamydial EB are antigenically complex and express a number of distinct antigens.[29] If the immune response to chlamydia is both protective and destructive, immunization with whole EB may well induce both types of response. A purified antigen preparation, therefore, might be more successful in eliciting protection than vaccines using the antigenically complex whole organism. We are currently exploring the efficacy of oral immunization with recombinant bacteria that express a single specific chlamydial antigen.[30] Finally, it may be that enteric immunization alone may not be able to prime the conjunctiva sufficiently to completely resist subsequent ocular challenge, and studies assessing topical ocular and combined oral and ocular immunization are planned.

Key words: chlamydia, trachoma, mucosal immunity, vaccination, monkeys

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References

1. Taylor HR, Johnson SL, Prendergast RA, Schachter J, Dawson CR, and Silverstein AM: An animal model of trachoma II. The importance of repeated reinfection. Invest Ophthalmol Vis Sci 23:507, 1982.
2. Taylor HR, Prendergast RA, Dawson CR, Schachter J, and Silverstein AM: Animal model of trachoma: III. The necessity of repeated exposure to live chlamydia. *In* Chlamydial Infections. Mardh PA, Holmes KK, Oriel JD, Piot P, Schachter J, editors. Amsterdam, Elsevier Biomedical Press, 1982, pp. 387–390.
3. Taylor HR: Report of a workshop: research priorities for the control of trachoma. J Infect Dis 152:383, 1985.
4. Grayston JT and Wang S: New knowledge of chlamydiae and the diseases they cause. J Infect Dis 132:87, 1975.
5. Schachter J and Dawson CR: Human chlamydial infections. Littleton, Massachusetts, PSG Publishing Company, 1978, pp. 83–88.
6. Collier LH and Blyth WA: Immunogenicity of experimental trachoma vaccines in baboons. II. Experiments with adjuvants and tests of cross-protection. J Hyg (Camb) 64:529, 1966.
7. Grayston JT, Kim KSW, Alexander ER, and Wang SP: Protective studies in monkeys with trivalent and monovalent trachoma vaccines. Chapter 31. *In* Trachoma and Related Disorders. Nichols RL, editor. Amsterdam, Excerpta Medica, 1971, pp. 377–385.
8. Fraser CEO: The owl monkey (*Aotus trivirgatus*) as an animal model in trachoma research. Lab Anim Sci 26:1138, 1976.
9. Clements C and Dhir SP: Long-term follow-up study of a trachoma vaccine trial in villages of Northern India. Am J Ophthalmol 87:350, 1979.
10. Schachter J and Caldwell HD. Chlamydiae. Ann Rev Microbiol 34:285, 1980.

11. Svennerholm AM, Gothefors L, Sack DA, Bardhan-PK, and Holmgren J: Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes. Bull WHO 62:909, 1984.
12. Zhang P, Pierce NF, Silverstein AM, and Prendergast RA: Conjunctival immunity: compared effects of ocular or intestinal immunization in rats. Invest Ophthalmol Vis Sci 24:1411, 1983.
13. Taylor HR, Pierce NF, Zhang P, Schachter J, Silverstein AM, and Prendergast RA: Secretory immune cellular traffic between the gut and the eye. *In* Advances in Immunology and Immunopathology of the Eye. O'Connor GR, Chandler JW, editors. New York, Masson Publishing, 1985, pp. 208–211.
14. Nichols RL, Murray ES, and Nisson PE: Use of enteric vaccines in protection against chlamydial infections of the genital tract and the eye of the the guinea pigs. J Infect Dis 138:742, 1978.
15. Caldwell HD, Kromhout J, and Schachter J: Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect Immun 31:1161, 1981.
16. Houghten RA, Engert RF, Outresh JM, Hoffman SR, and Klipstein FA: A completely synthetic toxoid vaccine containing *Escherichia coli* heat-stable toxin and antigenic determinants of the heat-labile toxin B subunit. Infect Immun 48:735, 1985.
17. Taylor HR, Prendergast RA, Dawson CR, Schachter J, and Silverstein AM: An animal model for cicatrizing trachoma. Invest Ophthalmol Vis Sci 21:422, 1981.
18. Taylor HR, Agarwala N, and Johnson S: Detection of experimental *Chlamydia trachomatis* eye infection in conjunctival smears and in tissue culture with a fluorescein-conjugated monoclonal antibody. J Clin Microbiol 20:391, 1984.
19. Wang SP and Grayston JT: Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Am J Ophthalmol 70:367, 1970.
20. Young E and Taylor HR: Immune mechanisms in chlamydial eye infection: cellular immune response in chronic and acute disease. J Infect Dis 150:745, 1984.
21. Stuart ES and MacDonald AB: Identification of two fatty acids in a group determinant of *Chlamydia trachomatis*. Current Microbiology 11:123, 1984.
22. Pierce NF: The role of antigen form and function in the primary and secondary intestinal immune responses to cholera toxin and toxoid in rats. J Exp Med 148:195, 1978.
23. Pierce NF and Koster FT: Priming and suppression of the intestinal immune response to cholera toxoid/toxin by parenteral toxoid in rats. J Immunol 124:307, 1980.
24. Keren DF, McDonald RA, Scott PJ, Rosner AM, and Strubel E: Effect of antigen form on local immunoglobulin A memory response of intestinal secretions to *Shigella flexneri*. Infect Immun 47:123, 1985.
25. Whittum-Hudson J, Taylor HR, Farazdaghi M, and Prendergast RA: Immunohistochemical study of the local inflammatory response to chlamydial ocular infection. Invest Ophthalmol Vis Sci 27:64, 1986.
26. Rank RG, White HJ, and Barron AL: Humoral immunity in the resolution of genital infection in female guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. Infect Immun 26:573, 1979.
27. Johnson AP: Genital infection of marmosets with *Chlamydia trachomatis*. *In* Chlamydial Infections, Mardh PA, Holmes KK, Oriel JD, Piot P, Schachter J, editors. Amsterdam, Elsevier Biomedical Press, 1982, pp. 395–398.
28. Byrne GI: Requirements for ingestion of *Chlamydia psittaci* by mouse fibroblasts (L cells). Infect Immun 14:645, 1976.
29. MacDonald AB: Antigens of *Chlamydia trachomatis*. Rev Infect Dis 7:731, 1985.
30. Nano FE and Caldwell HD: Expression of the chlamydial genus-specific lipopolysaccharide epitope in *Escherichia coli*. Science 228:742, 1985.

STIC-ILL

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Immunization against chlamydial genital infection in guinea pigs with UV-inactivated and viable chlamydiae administered by different routes.
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Immunization against Chlamydial Genital Infection in Guinea Pigs with UV-Inactivated and Viable Chlamydiae Administered by Different Routes

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Female guinea pigs were immunized with viable or UV light-inactivated chlamydiae (agent of guinea pig inclusion conjunctivitis), belonging to the species *Chlamydia psittaci*, by intravenous, subcutaneous, oral, or ocular routes. All animals were then inoculated vaginally with viable chlamydiae to determine the extent of protection against challenge infection induced by the various regimens. The course of genital infection was significantly reduced in intensity in all groups of animals except the unimmunized controls and those animals immunized orally with inactivated antigen. Guinea pigs immunized with viable antigen were more likely to develop resistance to challenge infection and, in general, had a significantly greater degree of protection than animals immunized with inactivated antigen. No one route seemed superior in producing a protective response. Animals in all groups demonstrating protection developed serum and secretion immunoglobulin G antibody responses to chlamydiae. Lymphocyte proliferative reactions to chlamydial antigen were variable among groups. Immunoblot analysis of serum and secretions indicated a wide range of antibody specificities, but most protected animals produced antibodies to the major outer membrane protein, lipopolysaccharide, and the 61-kilodalton protein. No definitive associations could be made between the increased ability of immunization with viable organisms to produce resistance to challenge infection and a particular immune parameter. These data indicate that viable chlamydiae given by various routes are able to induce a strong immune response which can provide resistance against reinfection in some cases or at least reduce the degree of infection to a greater degree than inactivated antigen. However, complete resistance to genital tract infection may be difficult to obtain and alternate immunization strategies may have to be developed.

Genital infections caused by *Chlamydia trachomatis* remain a major problem despite the availability of effective chemotherapeutic agents. This is particularly true in females, in that irreversible tubal damage may be produced before the infection is diagnosed and treated. Thus, it would be most appropriate to develop an immunization procedure to prevent infection from occurring.

We have been studying the basic immune mechanisms responsible for resolving a primary chlamydial genital infection and providing resistance to reinfection by using guinea pigs infected with the *C. psittaci* agent of guinea pig inclusion conjunctivitis (GPIC). Despite the fact that this organism is classified as *C. psittaci*, the disease in guinea pigs has been demonstrated to closely resemble human chlamydial genital infections with regard to pathogenesis, pathology, and immunology (1, 20). We have found that both antibody and cell-mediated immune (CMI) mechanisms are required for resolution of the infection (21, 27) as well as resistance to challenge infection (22, 26). Of major concern, however, was our finding that complete resistance to reinfection following a primary infection was relatively short in duration. By 2 months after resolution of the primary infection, animals became reinfected upon challenge but had a markedly less severe infection as well as one of shorter duration. This "partial" immunity persisted for up to about 2 years (2, 25). The loss of complete resistance was associated with the decline of immunoglobulin G (IgG) antibodies in serum and IgG and IgA in genital secretions as well as a decline in CMI response as determined by lymphocyte proliferation responses to GPIC antigen (25). Humans, too, can become reinfected, and some evidence suggests that immunity does develop but is also short-lived (9). Thus, the problem becomes how one can induce a long-lasting protective immune response in the genital tract when a primary infection, usually the strongest immunizing event, does not itself elicit long-term protection. To date, no effective vaccines against genital infections with any of the genital pathogens have been developed in humans or animal models. To further complicate the situation, it has been suggested, although not proven in humans or in models of ascending infection, that delayed-type hypersensitivity mechanisms may actually exacerbate the disease process in the fallopian tubes (17, 18). There is certainly precedent for this phenomenon in chlamydial infections of the eye (28, 29).

In this study, we explored several different routes of immunization with either viable or inactivated antigen in the guinea pig model to determine whether immunity to reinfection could be elicited. The results of challenge infection were correlated to levels of different immune parameters prior to challenge. These parameters included total anti-GPIC and component-specific antibody responses in serum and secretions and lymphocyte proliferation to GPIC as a measure of cell-mediated immunity.

MATERIALS AND METHODS

Experimental animals. Female Hartley strain guinea pigs, weighing 450 to 500 g, were obtained from Sasco Laboratories, Omaha, Nebr. All animals were housed individually in cages covered with fiberglass filters and were given food and

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water ad libitum. The room was maintained on a 12:12 light:dark cycle.

Infection of guinea pigs with chlamydiae. The GPIC agent for infection was grown in HeLa cell cultures (7). Aliquots of host cell-free chlamydiae were frozen in a sucrose-phosphate buffer (2-SP) at −70°C until needed. Animals were infected by intravaginal inoculation of 0.05 ml of the GPIC suspension which contained 10^7 to 10^8 inclusion-forming units (IFU) (25). Animals were monitored for the course of the infection by assessing the percentage of chlamydial inclusions on a vaginal scraping stained with Giemsa and by the isolation of organisms in McCoy cell cultures from cervical swabs (25). The cultures were not quantified by were merely scored as positive or negative for chlamydiae. The latter method has been found to be the more sensitive of the two assay systems (25).

Immunization of guinea pigs with GPIC. Viable organisms for immunization were grown in McCoy cells and were stored cell-free in 2-SP at −70°C until needed. For injection, the organisms were diluted in phosphate-buffered saline (PBS). Inactivated GPIC was prepared by treating McCoy cell-grown and Percoll-purified elementary bodies with UV light (G30T8 UV lamp, model J225, Blak Ray) at a distance of 10 cm for 3 h. When such preparations were assessed for viable organisms in cell culture, none were detected. The UV-inactivated chlamydiae were suspended in PBS for immunization purposes.

Intravenous immunization was accomplished by injecting methoxyflurane-anesthetized guinea pigs in one of the superficial veins on the dorsal aspect of the pinna of the ear by using a 30-gauge, 0.5-in. needle bent at a 45° angle. Care was taken with the live material to swab the area with 70% ethanol after the injection. Each animal received either 4×10^7 IFU of viable organisms or 100 μg of inactivated GPIC, each contained in 0.1 ml of PBS.

Animals were injected subcutaneously in two sites in the inguinal area with 0.25 ml of PBS each for a total of 2×10^8 IFU of viable GPIC or 500 μg of inactivated GPIC. In one experiment, animals were immunized subcutaneously with 500 μg of inactivated GPIC suspended in an equal volume of Freund complete adjuvant. For the booster inoculations, the GPIC antigen was suspended in Freund incomplete adjuvant. Animals were injected with 0.25 ml of the suspension at four sites in the axial and inguinal areas.

Oral immunization was performed by anesthetizing guinea pigs with methoxyflurane and passing a piece of Tygon tubing (outside diameter, 2 mm; length, 8 cm) connected to an 18-gauge, 1.5-in. needle about 6 cm into the oral cavity. Each animal received 0.5 ml of PBS containing either 2×10^8 IFU of viable chlamydiae or 500 μg of inactivated organisms.

In one experiment, guinea pigs were immunized by a single ocular infection with 5×10^6 IFU as previously described (23).

All animals (except those ocularly immunized) were given a primary inoculation followed by two booster inoculations at 2-week intervals and were challenged 2 weeks after the last immunization by intravaginal inoculation. During the immunization period all animals, particularly those given live organisms, were monitored for the development of ocular (except the ocular-immunized group) or genital infections. If animals were positive for either, they were eliminated from the study.

Determination of antibody levels. Sera and genital secretions were obtained from guinea pigs as previously described (22, 27) and stored at −20°C until all specimens in the experiment were collected. Serum IgG was measured by an enzyme-linked immunosorbent assay using HeLa-grown GPIC elementary bodies as the antigen and peroxidase-labeled rabbit anti-guinea pig IgG (heavy- and light-chain specific) (ICN ImmunoBiologicals, Lisle, Ill.) (23). IgA in genital secretions was determined by a similar assay except that rabbit anti-guinea pig IgA (α-chain specific) (ICN) was used, followed by peroxidase-labeled goat anti-rabbit IgG (heavy- and light-chain specific) (ICN) (23).

Immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretic transfer of chlamydial proteins to nitrocellulose membranes, and immunoblotting assays were performed as previously described (2). Nitrocellulose membranes were blocked by using a solution containing 150 mM NaCl–10 mM Tris–0.5% (wt/vol) nonfat dry milk (Carnation), pH 7.4. Guinea pig serum specimens were diluted 1:500 to 1:1,000. Antibody binding was localized by using rabbit anti-guinea pig IgG (heavy- and light-chain specific) (Miles Laboratories, Inc., Naperville, Ill.) followed by radioiodinated goat anti-rabbit IgG (Cooper Biomedical, Inc., West Chester, Pa.) and autoradiography.

Individual immunoreactive bands were judged to be present or absent in a blinded fashion. Molecular weights were assigned to minor bands by measuring their migration on autoradiograms and comparing them to a plot of migration versus molecular weight. Major bands of known molecular mass (39-kilodalton [kDa] major outer membrane protein [MOMP], 61 kDa, 15 kDa) were used as standards.

Determination of cell-mediated immune response. CMI activity in immunized animals was assessed by the proliferative response of peripheral blood lymphocytes to GPIC antigen and concanavalin A (Sigma Chemical Co.) 2 to 3 days before challenge infection (25). Four milliliters of blood was obtained by cardiac puncture, and the blood was mixed with sodium citrate. The blood was diluted with 3 volumes of RPMI 1640 and centrifuged at $400 \times g$ for 40 min over Histopaque (specific weight, 1.077 g/ml) (Sigma). Peripheral blood mononuclear cells were collected from the interface, washed, and placed into microculture at 2×10^5 cells per well in RPMI 1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 2 mM glutamate, and penicillin (100 U/ml) and streptomycin (100 mg/ml). UV-inactivated HeLa-grown GPIC antigen (7) was added to triplicate wells at 16 μg/ml, and concanavalin A was similarly added at 2.5 μg/ml. Cultures were labeled with 1 μCi of [^{3}H]thymidine per ml over the final 24 h of a 5-day incubation at 37°C in 5% CO_2. The results were expressed as the mean counts per minute of cultures from five animals.

RESULTS

Course of challenge infections. In order to determine whether protection against a genital tract infection could be elicited with an inactivated antigen, an immunizing regimen was used which incorporated the use of Freund complete and incomplete adjuvants with UV-inactivated GPIC in the immunization of five guinea pigs. Another group of five guinea pigs was given a single ocular infection with GPIC as a positive control, since it has been previously shown that this would induce protection against a genital infection (14; R. G. Rank, unpublished data). A third group remained unimmunized to serve as an infection control.

Both immunized groups demonstrated a significant degree of protection when compared to challenged unimmunized controls ($P < 0.001$, according to a two-factor [days, treatment] analysis of variance with repeated measures on one

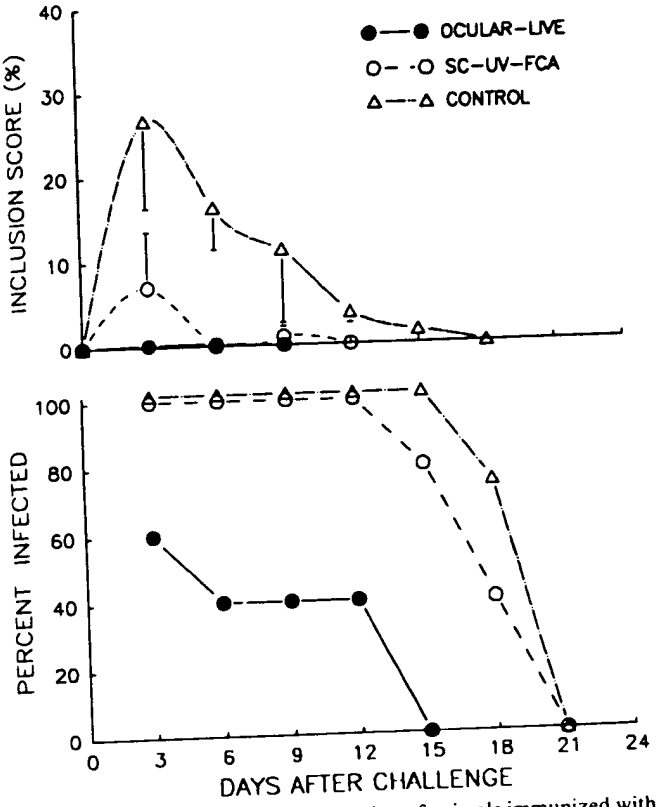

FIG. 1. Results of challenge infection of animals immunized with UV-inactivated GPIC in Freund complete adjuvant (FCA) or by a single prior ocular infection. (A) Coures of challenge infection as measured by inclusion score. (B) Course of infection as determined by percentage of animals infected as assessed by isolation of GPIC from cervical swabs. SC, subcutaneous.

factor [days]) when the course of their infections was assessed by the percentage of inclusion-bearing cells in vaginal scrapings (Fig. 1). When the infection course was monitored by isolation of chlamydiae from cervical swabs, two of the five ocular-infected animals were resistant to reinfection, and the infections of the other three animals were resolved by day 15. All animals immunized with adjuvant and inactivated antigen became infected upon challenge and did not resolve until day 21 even though the infection was less intense as judged by inclusion scores. Thus, the length of the infection in the group immunized with inactivated antigen was unaltered despite the development of high titers of serum IgG (>10,240), secretion IgG (5,120) and IgA (1,470), and strong lymphocyte proliferation reactions to GPIC antigen (5,741 cpm) in guinea pigs immunized with inactivated antigen.

Since the data from this first experiment suggested that immunization with inactivated antigen was able to provide a degree of protection but that actual infection at a different site was somewhat more effective, we conducted a series of experiments to compare the effectiveness of different routes of immunization with either viable or UV-inactivated antigen without adjuvant. Each route was assessed in two to three experiments with five animals per group. The data from all experiments are summarized in Fig. 2 (inclusion scores) and 3 (chlamydial isolation). Intravenous, subcutaneous, and subcutaneous-oral immunization with inactivated antigen were able to significantly ($P < 0.001$) reduce the level of the

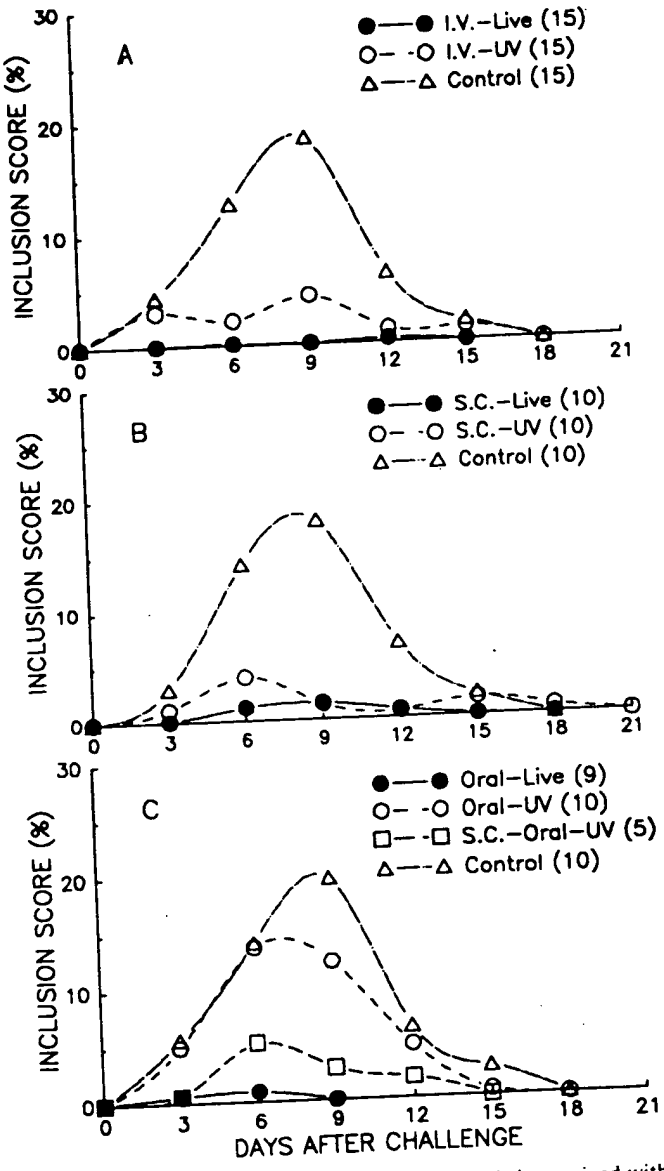

FIG. 2. Course of infection in groups of animals immunized with either viable or UV-inactivated GPIC by various routes as measured by inclusion score. I.V., Intravenous; S.C., subcutaneous.

challenge infection when compared to that of controls (Fig. 2). Oral immunization with inactivated antigen, in general, did not produce any protective effect, although two animals had negative inclusion scores upon challenge. Immunization with viable chlamydiae by all routes significantly shortened the course and markedly lowered the intensity of the challenge infection. In many animals demonstrating protection, no inclusions could be detected at any time point during the observation period.

When cervical swabs were assayed for viable chlamydiae, few animals were found to be uninfected after challenge. All animals immunized with inactivated antigen became reinfected, and despite the low inclusion scores in many animals, no differences were seen between immunized and control guinea pigs with regard to the length of the challenge infection (Fig. 3). Only animals receiving viable organisms demonstrated resistance to reinfection. Of these, intrave-

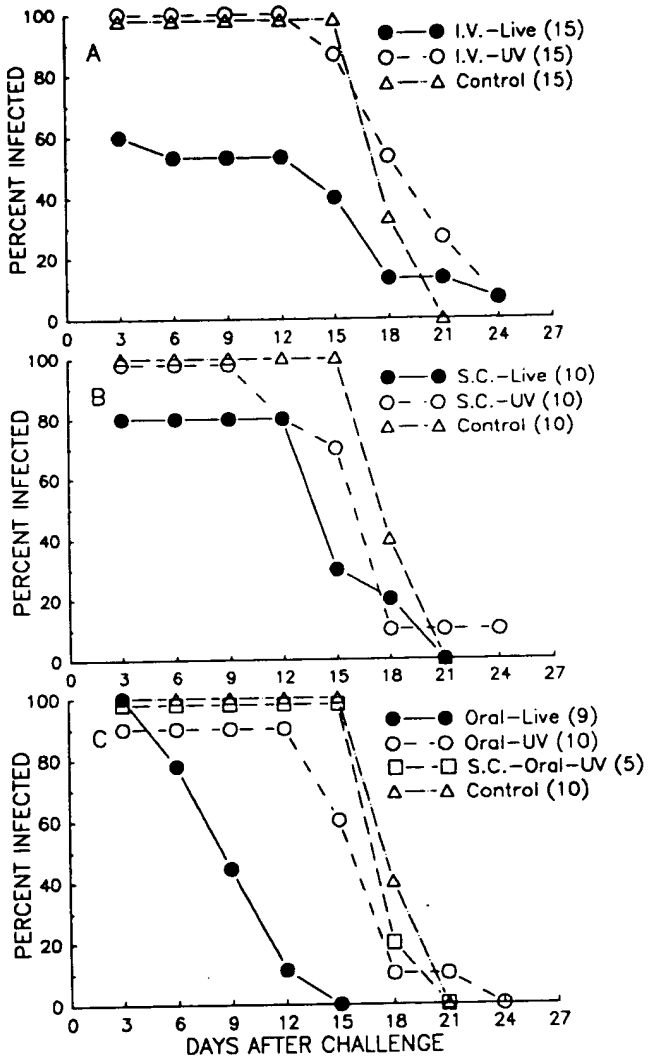

FIG. 3. Course of infection in groups of animals immunized with either viable or UV-inactivated GPIC by various routes as measured by isolation of GPIC from cervical swabs. I.V., Intravenous; S.C., subcutaneous.

nous inoculation of viable GPIC produced resistance in 6 of 15 guinea pigs. Animals immunized orally with live organisms resolved the challenge infection more quickly than control guinea pigs or guinea pigs immunized with inactivated antigen. However, some animals immunized with viable GPIC remained infected as long as unimmunized controls.

The difference in protective capacity of the live versus inactivated vaccines was analyzed by determining the length of infections, as determined by isolation of GPIC, of animals receiving live or inactivated preparations. This information was combined for all animals immunized with viable organisms and all animals immunized with inactivated organisms, regardless of the route, and the percentage of each group with infections of n days in length was determined (Fig. 4). Animals immunized with live organisms had significantly shorter infections (8.1 ± 6.6) than those animals immunized with inactivated organisms (15.2 ± 4.3) or unimmunized controls (16.0 ± 1.5) ($P < 0.0001$ according to a one-tailed t test).

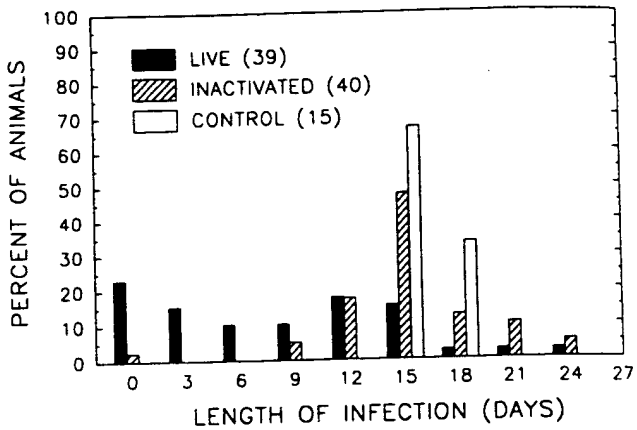

FIG. 4. Length of infections of guinea pigs immunized with inactivated or live organisms or those remaining untreated. The total number of animals for each group is given in parentheses, and each bar represents the percentage of that total which had infections of n days. Infection was determined by isolation of organisms from cervical swabs obtained at 3-day intervals.

Immune parameters. Various immune parameters were determined on all animals 1 or 2 days prior to challenge infection in an attempt to correlate protection or resistance with a particular immune response. Figure 5 represents a single experiment in which five animals each were immunized with viable or inactivated antigen by intravenous, subcutaneous, or oral routes. All groups with the exception of the group immunized orally with inactivated antigen produced high serum and secretion IgG antibody responses. IgA antibody levels in genital secretions were low throughout except for animals immunized subcutaneously with inactivated antigen. When peripheral blood lymphocytes were tested for proliferation to GPIC antigen, the best responses were seen in subcutaneously immunized guinea pigs, either with live or inactivated antigen. Although results were not shown in Fig. 5, the two animals in the group immunized orally with inactivated antigen which were immune were the only animals in that group to have elevated serum and secretion IgG as well as lymphocyte proliferation responses. It is interesting that nonspecific lymphocyte proliferation as measured by the response to concanavalin A was significantly greater ($P < 0.05$ according to a one-factor analysis of variance with Scheffe test for groups with significant differences) in the animals infected subcutaneously when compared to all groups except for intravenously infected guinea pigs (data not shown).

When a correlation matrix (Pearson's) was performed on data from 89 animals included in this study for which antibody and proliferative responses were available, the only parameter which correlated with low inclusion scores upon challenge was the level of serum IgG antibody. Thus the higher the titer of serum IgG antibody to GPIC, the lower the inclusion scores in challenged animals. Although the level of secretion IgG was strongly correlated to that of serum IgG, no association was seen between the level of infection and the titer of secretion IgG.

Antigen-specific antibody response. The antigen-specific antibody response in serum was also assessed prior to challenge infection to determine if the presence or absence of an antibody response to a particular antigen could be associated with protection or susceptibility to challenge infection. Each serum was evaluated for the presence of IgG

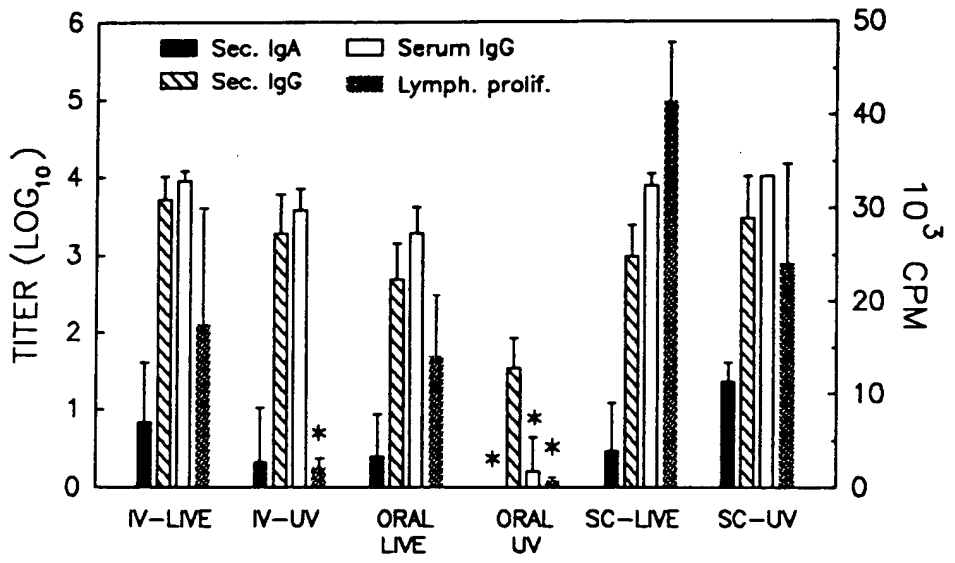

FIG. 5. Immune response parameters from guinea pigs immunized by different routes with either live or UV-inactivated GPIC. Data are from a single experiment so that comparisons among groups can be more readily identified. *, Significant difference of at least $P < 0.05$ according to a one-factor analysis of variance with a Scheffe test for significant differences. Sec., Secretion; I.V., intravenous; S.C., subcutaneous; Lymph. prolif., lymphocyte proliferation.

antibody to each of 13 different antigens. With the exception of most guinea pigs immunized orally with inactivated antigen which had negligible antibody responses, all animals produced serum antibodies to the 61-kDa protein and a very high percentage responded to MOMP and lipopolysaccharide (LPS) (Table 1). The 61-kDa protein is an outer membrane protein analogous to the cysteine-rich outer membrane protein of *C. trachomatis* (6) although a Sarkosyl-soluble protein comigrates with it in preparations of whole elementary bodies. The 61-kDa Sarkosyl-soluble protein is probably the same as the 57-kDa delayed-hypersensitivity protein reported by Morrison et al. (11). To differentiate between the Sarkosyl-soluble and Sarkosyl-insoluble moieties appearing in the 61-kDa area, some sera in each group were assessed for antibody activity to the two Sarkosyl fractions by immunoblot analysis. Virtually all animals responded to the 61-kDa protein in both soluble and insoluble fractions (data not shown) as has been seen before (2). Most animals also responded to the 47-kDa moiety. The serum antibody responses to other antigens were variable among and within the different immunization regimens.

One interesting observation is that sera from guinea pigs immunized orally with viable GPIC were consistently positive only in their reactions to MOMP, LPS, and the 61-kDa protein. Moreover, the two protected animals in the group immunized orally with inactivated GPIC were the only animals in that group to respond to the 61-kDa protein (both animals), MOMP (1 animal), and LPS (both animals).

Similar results were obtained upon determination of the secretion IgG antibody response although not a large number of animals were tested. Antibodies to MOMP, LPS, and the 61-kDa protein were present in the majority of the animals. Even the animals immunized orally with inactivated GPIC had a high response rate to the 61-kDa protein and about a 50% response rate to MOMP. The remainder of the reactions among all immunization groups to other antigens was variable.

DISCUSSION

The data presented in this study indicate that immunization of guinea pigs with UV-inactivated GPIC elementary

TABLE 1. Serum IgG antibody responses to specific chlamydial antigens as determined by immunoblot analysis

Antigen	% of animals responding to antigen (no. of animals tested)[a]							
	Ocular (5)	i.v.-live (15)	i.v.-UV (15)	s.c.-live (10)	s.c.-UV (10)	Oral-live (9)	Oral-UV (10)	s.c.-oral (5)
84 kDa	100	87	80	80	90	33	10	0
72 kDa	20	53	33	50	50	11	0	0
61 kDa	100	100	100	100	100	100	20	100
52 kDa	20	47	0	50	0	11	0	0
47 kDa	100	100	93	60	100	89	50	80
MOMP	100	100	100	90	100	100	10	80
35 kDa	0	47	93	40	90	11	0	20
33 kDa	100	73	7	40	20	11	0	20
31 kDa	40	73	20	40	50	11	0	0
27 kDa	80	100	87	80	90	22	0	40
19 kDa	0	100	87	0	90	11	0	40
15 kDa	100	47	53	100	80	67	70	40
LPS kDa	100	100	93	100	100	100	20	100

[a] Regimens: i.v., intravenous; s.c., subcutaneous.

bodies is able to provide a significant measure of protection against a challenge infection in the genital tract. This protection is, however, only reflected in a decreased intensity of the challenge infection and not in an increased resistance to infection nor in an abbreviated infection course; i.e., all animals given inactivated antigen became infected, and these infections were unaltered in duration compared to controls. The route of immunization did govern to some extent the development of protection against challenge. Both intravenous and subcutaneous routes were effective in inducing a protective immune response as judged by a decrease in inclusion scores, but oral immunization conferred protection in only a small proportion of animals. Only when oral immunization was preceded by a subcutaneous primary injection did immunity result. The lack of significant protection by oral immunization with inactivated organisms was somewhat unexpected since induction of an immune response with an inert antigen via a mucosal pathway has been shown to elicit a good response at other mucosal sites (10).

Since it has been previously observed by others that prior genital, ocular (8, 14), or oral (16) infection results in resistance to reinfection at a distant site in a certain percentage of animals, we compared the effect of inoculation of guinea pigs with viable GPIC by the intravenous, subcutaneous, ocular, and oral routes to immunization with inactivated antigen via these same routes (except the ocular route). Interestingly, injection of viable organisms by the intravenous and subcutaneous routes resulted in 40 and 20%, respectively, of the animals being completely resistant to reinfection. Although oral inoculation of live GPIC did not elicit resistance to reinfection, the course of infection in most animals was considerably shorter than in control animals or animals immunized with inactivated antigen by any route. A smaller percentage of animals in each group had challenge infections of normal duration. Thus, immunization with viable organisms produced a significantly stronger immunity than immunization with inactivated antigen. Murray and Radcliffe (15) reported similar findings in attempts to immunize against ocular GPIC infection. In their studies, Formalin-inactivated antigens were found to be ineffectual in inducing immunity as judged by inclusion scores, while intraperitoneal injection of viable GPIC was able to reduce the level of subsequent ocular challenge infection. Moreover, Taylor et al. (30) also found that orally administered live but not UV-inactivated *C. trachomatis* serovar L_2 was able to induce a protective response against serovar B challenge in their primate model for trachoma.

When the immune response of the immunized guinea pigs was determined prior to challenge, a correlation was seen between the level of serum IgG and reduction in infection intensity, but no factors were found which could be associated with the increase in incidence of resistance to infection and the shortened duration of infection seen in animals given viable GPIC. Both intravenous and oral route groups given inactivated antigen had significantly lower lymphocyte proliferation reactions, but inactivated antigen administered subcutaneously induced a very strong proliferative response with no apparent difference in the kinetics of the challenge infection. The strongest T-cell response occurred in the group injected subcutaneously with viable GPIC. This group also had an increase in the nonspecific T-cell proliferative response, but the resulting infection course upon challenge was not remarkably different from the other groups given viable organisms.

The only factor positively identified with a protective response, as measured by inclusion scores, was the ability of the immunization regimen to induce the production of serum IgG. That serum antibody plays an important role in immunity to reinfection has been demonstrated previously when we were able to elicit a reduction in infection intensity by the passive transfer of immunoglobulin from immune animals to naive guinea pigs (24). Moreover, animals deficient in antibody but competent in CMI response to GPIC became reinfected upon challenge with infections resembling a primary GPIC genital infection (22).

It is quite likely that antibody in secretions is responsible for reducing the level of infection in all animals demonstrating some degree of protection. As would be expected, antibodies to a wide range of antigens were detected by immunoblot. The only antibody responses which were consistent among protected animals were directed against MOMP, LPS, and the 61-kDa protein(s), which would suggest that protective epitopes are contained on these moieties. Monoclonal antibodies to MOMP have been shown to neutralize infectivity both in vivo (31, 32) and in vitro (5, 19). We have demonstrated that MOMP, purified by using a nondenaturing detergent extraction, is capable of inducing a protective response against GPIC genital infection in guinea pigs (B. E. Batteiger, R. G. Rank, and L. S. F. Soderberg, unpublished data).

The reasons for the difference in protective capacity of live- versus inactivated-antigen immunization regimens are not clear, especially since UV inactivation does not alter the ability of the organism to enter the host cell or resist lysosomal fusion (4, 12). It is certainly possible that the live infection generates quantitatively more antigen by nature of replication, but one might expect that a difference should be reflected in the level of antibody and lymphocyte proliferative responses, which was not the case. More likely possibilities are that either different antigens are expressed by the dividing organism or that macrophage processing of a viable organism with the ability to replicate intracellularly results in a qualitatively different immune response, particularly with respect to the T-cell response. This concept is not without precedent. Müller and Louis (13) have reported that protective T-cell clones could be generated in vitro by using viable *Leishmania* organisms as the antigen, but they could not be induced with inactivated antigen. In fact, T-cell clones elicited with inactivated antigen were more likely to induce an immunopathological but not protective response.

The significance of this study is that a certain degree of immunity can be produced by immunization with inactivated antigen and to a somewhat greater extent with viable chlamydiae. Aside from the impracticality of immunizing with viable chlamydiae in human subjects, an important point to consider is that none of the regimens tested could reliably prevent challenge infection. In fact, it should be noted that actual genital infection, which should be the strongest immunizing event, results only in complete immunity to reinfection for a very short period of time, with animals becoming reinfected if challenged as soon as 50 days after resolution of a primary infection. Therefore, it is reasonable that an objective other than complete prevention of genital infection should be pursued. Since the major morbidity associated with genital infection is the development of salpingitis with resultant tubal obstruction, an appropriate immunization strategy would be the prevention of ascending infection but not necessarily the prevention of lower-genital-tract infection. The feasibility of this approach has been suggested by a study of recurrent gonococcal infection (3). Women who had pelvic inflammatory disease (PID) and were reinfected in the lower genital tract with the same

serotype had no recurrent PID. However, women who were reinfected with a different serotype had a 50% incidence of PID. Thus, the primary infection was able to prevent salpingitis upon challenge infection but not able to prevent infection of the lower genital tract. Since the data in the guinea pig model indicate that the level of infection can be reduced in immunized animals for relatively long periods of time, this strategy is a reasonable possibility.

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LITERATURE CITED

1. **Barron, A. L.** 1982. Contributions of animal models to the study of human chlamydial infections, p. 357–366. *In* P.-A. Mardh, J. Schachter, P. Piot, and D. Taylor-Robinson (ed.), Chlamydial infections. Elsevier Biomedical Press, Amsterdam.
2. **Batteiger, B. E., and R. G. Rank.** 1987. Analysis of the humoral immune response in chlamydial genital infection in guinea pigs. Infect. Immun. **55**:1767–1773.
3. **Buchanan, T. M., D. A. Eschenbach, J. S. Knapp, and K. K. Holmes.** 1980. Gonococcal salpingitis is less likely to recur with *Neisseria gonorrhoeae* of the same principal outer membrane protein antigenic type. Am. J. Obstet. Gynecol. **138**:978–980.
4. **Byrne, G. I., and J. W. Moulder.** 1978. Parasite-specified phagocytosis of *Chlamydia psittaci* and *Chlamydia trachomatis* by L and HeLa cells. Infect. Immun. **19**:598–606.
5. **Caldwell, H. D., and L. J. Perry.** 1982. Neutralization of *Chlamydia trachomatis* infectivity with antibodies to the major outer membrane protein. Infect. Immun. **38**:745–754.
6. **Hatch, T. P., I. Allan, and J. H. Pearce.** 1984. Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. J. Bacteriol. **157**:13–20.
7. **Hough, A. J., Jr., and R. G. Rank.** 1988. Induction of arthritis in C57Bl/6 mice by chlamydial antigen: effect of prior immunization or infection. Am. J. Pathol. **130**:163–172.
8. **Howard, L. V., M. P. O'Leary, and R. L. Nichols.** 1976. Animal model studies of genital chlamydial infections. Immunity to re-infection with guinea-pig inclusion conjunctivitis agent in the urethra and eye of male guinea-pigs. Br. J. Vener. Dis. **52**: 261–265.
9. **Katz, B. P., B. E. Batteiger, and R. B. Jones.** 1987. Effect of prior sexually transmitted disease on the isolation of *Chlamydia trachomatis*. Sex. Transm. Dis. **14**:160–164.
10. **Mestecky, J.** 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J. Clin. Immunol. **7**:265–276.
11. **Morrison, R. P., R. J. Belland, K. Lyng, and H. D. Caldwell.** 1989. Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein. J. Exp. Med. **170**:1271–1283.
12. **Moulder, J. W., T. P. Hatch, G. I. Byrne, and K. R. Kellogg.** 1976. Immediate toxicity of high multiplicities of *Chlamydia psittaci* for mouse fibroblasts (L cells). Infect. Immun. **14**: 277–289.
13. **Müller, I., and J. A. Louis.** 1989. Immunity to experimental infection with Leishmania major: Generation of protective L3T4+ T cell clones recognizing antigen(s) associated with live parasites. Eur. J. Immunol. **19**:865–871.
14. **Murray, E. S.** 1977. Review of clinical, epidemiological, and immunological studies of guinea pig inclusion conjunctivitis infection in guinea pigs, p. 199–204. *In* D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
15. **Murray, E. S., and F. T. Radcliffe.** 1967. Immunologic studies in guinea pigs with guinea pig inclusion conjunctivitis (Gp-ic) Bedsonia. Am. J. Ophthalmol. **63**:1263–1269.
16. **Nichols, R. L., E. S. Murray, and P. E. Nisson.** 1978. Use of enteric vaccines in protection against chlamydial infections of the genital tract and the eye of guinea pigs. J. Infect. Dis. **138**:742–746.
17. **Patton, D. L., and C.-C. Kuo.** 1989. Histopathology of *Chlamydia trachomatis* salpingitis after primary and repeated reinfections in the monkey subcutaneous pocket model. J. Reprod. Fertil. **85**:647–656.
18. **Patton, D. L., C.-C. Kuo, S.-P. Wang, and S. A. Halbert.** 1987. Distal tubal obstruction induced by repeated *Chlamydia trachomatis* salpingeal infection in pig-tailed macaques. J. Infect. Dis. **155**:1292–1299.
19. **Peeling, R., I. W. Maclean, and R. C. Brunham.** 1984. In vitro neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. Infect. Immun. **46**:484–488.
20. **Rank, R. G.** 1988. Role of the immune response, p. 217–234. *In* A. L. Barron (ed.), Microbiology of chlamydia. CRC Press, Boca Raton, Fl.
21. **Rank, R. G., and A. L. Barron.** 1983. Effect of antithymocyte serum on the course of chlamydial genital infection in female guinea pigs. Infect. Immun. **41**:876–879.
22. **Rank, R. G., and A. L. Barron.** 1983. Humoral immune response in acquired immunity to chlamydial genital infection of female guinea pigs. Infect. Immun. **39**:463–465.
23. **Rank, R. G., and A. L. Barron.** 1987. Specific effect of estradiol on the genital mucosal antibody response in chlamydial ocular and genital infections. Infect. Immun. **55**:2317–2319.
24. **Rank, R. G., and B. E. Batteiger.** 1989. Protective role of serum antibody in immunity to chlamydial genital infection. Infect. Immun. **57**:299–301.
25. **Rank, R. G., B. E. Batteiger, and L. S. F. Soderberg.** 1988. Susceptibility to reinfection after a primary chlamydial genital infection. Infect. Immun. **56**:2243–2249.
26. **Rank, R. G., L. S. F. Soderberg, M. M. Sanders, and B. E. Batteiger.** 1989. Role of cell-mediated immunity in the resolution of secondary chlamydial genital infection in guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. Infect. Immun. **57**:706–710.
27. **Rank, R. G., H. J. White, and A. L. Barron.** 1979. Humoral immunity in the resolution of genital infection in female guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. Infect. Immun. **26**:573–579.
28. **Taylor, H. R., S. L. Johnson, R. A. Prendergast, J. Schachter, C. R. Dawson, and A. M. Silverstein.** 1982. An animal model of trachoma II. The importance of repeated reinfection. Invest. Ophthalmol. Vis. Sci. **23**:507–515.
29. **Taylor, H. R., S. L. Johnson, J. Schachter, H. D. Caldwell, and R. A. Prendergast.** 1987. Pathogenesis of trachoma: the stimulus for inflammation. J. Immunol. **138**:3023–3027.
30. **Taylor, H. R., E. Young, A. B. MacDonald, J. Schachter, and R. A. Prendergast.** 1987. Oral immunization against chlamydial eye infection. Invest. Ophthalmol. Vis. Sci. **28**:249–258.
31. **Zhang, Y.-X., S. J. Stewart, and H. D. Caldwell.** 1989. Protective monoclonal antibodies to *Chlamydia trachomatis* serovar- and serogroup-specific major outer membrane protein determinants. Infect. Immun. **57**:636–638.
32. **Zhang, Y.-X., S. Stewart, T. Joseph, H. R. Taylor, and H. D. Caldwell.** 1987. Protective monoclonal antibodies recognize epitopes located on the outer membrane protein of *Chlamydia trachomatis*. J. Immunol. **138**:575–581.

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Systemic immunization with Hsp60 alters the development of chlamydial ocular disease.
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Systemic Immunization With Hsp60 Alters the Development of Chlamydial Ocular Disease

Roger G. Rank, Christopher Dascher,† Anne K. Bowlin,* and Patrik M. Bavoil†*

Purpose. To determine whether immunization with recombinant Hsp60 would exacerbate ocular pathology on challenge with viable chlamydial elementary bodies.

Methods. Guinea pigs were immunized either subcutaneously with recombinant Hsp60 or both subcutaneously with recombinant Hsp60 and ocularly with attenuated *Salmonella typhimurium* expressing the guinea pig inclusion conjunctivitis (GPIC) Hsp60 antigen. All animals were challenged in the conjunctiva with the agent of GPIC, and the degree of gross ocular pathology was determined. Immunoglobulin G (IgG) and immunoglobulin A (IgA) antibody titers to Hsp60 were measured in ocular secretions as a measure of the degree of immunization.

Results. In primary and challenge GPIC infection, the degree of gross ocular pathology was lower in the immunized group. The presence of high IgA and IgG antibody titers to Hsp60 in tears suggested that the response may have been modified by the presence of blocking antibodies that either may have removed the antigen quickly or prevented interaction with sensitized T cells. In contrast to subcutaneous immunization, the combined immunization regimen, consisting of subcutaneous recombinant Hsp60 followed by ocular inoculation of the attenuated *Salmonella*, resulted in no difference in gross pathology after reinfection of guinea pigs with GPIC.

Conclusions. These data indicated that the immunization with Hsp60 did not produce exacerbated disease on challenge with viable organisms; however, the data suggested that the route of administration, form of antigen, or both may be critical in the disease process. Invest Ophthalmol Vis Sci. 1995;36:1344–1351.

It has been well documented in the human[1,2] and in the primate[3] that trachoma results primarily from an immunopathologic response to repeated or chronic infection with *Chlamydia trachomatis*. Although the use of a whole elementary body vaccine for trachoma is not endorsed, it is of interest to determine those chlamydial components responsible for eliciting the pathologic response. By definition, these may not be the optimal components of a vaccine and should in fact be deleted from a potential vaccine.

At least one candidate for an antigen capable of eliciting such a response is the 57-kd heat shock protein (Hsp60) of *Chlamydia*. Watkins et al[4] originally reported that a Triton-X 100 extract of elementary bodies (EB) from the *C. psittaci* agent of guinea pig inclusion conjunctivitis (GPIC) could elicit a pathologic response in the eyes of previously infected guinea pigs that resembled the reaction to a primary GPIC infection. The guinea pig–GPIC model was found to be an excellent model for trachoma because repeated infection can produce a disease similar to that appearing in humans.[5] Morrison et al[6,7] later cloned and identified the active component of this extract as the chlamydial homologue of *Escherichia coli* GroEL. The homologous protein isolated from *C. trachomatis* was found also to be capable of inducing a delayed-type hypersensitivity (DTH) response in the eyes of previously infected monkeys.[8] Although it is not clear if there are other chlamydial antigens that can elicit a DTH response, it seems obvious from these experiments that any potential vaccine against *Chlamydia* should not include the Hsp60 protein.

However, one caveat of the above studies was that

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the response was always elicited with purified protein in a buffer containing Triton X-100, an artificial situation. No experiments have been reported in which animals sensitized to Hsp60 by prior immunization had more severe reactions when challenged with live EB. The purpose of this study was, thus, to determine whether immunization with Hsp60 would actually elicit an exacerbated pathologic response when animals were challenged in the eye with viable GPIC.

METHODS

Experimental Animals

Female Hartley strain guinea pigs, each weighing 450 to 500 g, were obtained from Sasco Laboratories (Omaha, NE). All animals were housed individually in cages covered with fiberglass filter tops and given food and water ad libitum. Unless stated otherwise, each experimental group routinely consisted of five animals. Experiments were repeated at least once. All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Culture of Chlamydiae and Assessment of Infection

Purification of GPIC for antigen and for infection purposes was performed according to standard practices previously described.[9] Stocks for infection were prepared in McCoy cells while antigen was prepared in HeLa cells. The infection was assessed by the collection of conjunctival scrapings using a dental plastic instrument and then staining with Giemsa.[10] The inclusion score was determined as the percentage of epithelial cells containing chlamydial inclusions.

Ultraviolet (UV) light-inactivated GPIC was prepared by irradiating a suspension of GPIC approximately 10 cm from a Germidical lamp (Westinghouse Sterilamp #63615L) for 2 hours with constant stirring. This procedure has been found to inactivate 100% of the organisms present.

Infection of Guinea Pigs

Guinea pigs were infected in the conjunctiva by instilling 20 μl of GPIC suspended in sucrose–phosphate buffer (2-SP)[11] directly into the conjunctival sac. In some cases, only one eye was inoculated. In the assessment of infection in these animals, both eyes were observed for the development of disease, but in no case was contamination of the uninoculated eye detected. Infection doses were generally approximately 10^6 inclusion-forming units of GPIC. This dose, though perhaps unrealistically high in comparison to humans, was used to ensure a larger amount of antigen to provide the maximum chance to elicit the pathologic response.

Purification of rHsp60

Recombinant HSP60 (rHsp60) expressed by JM109 (pGP57)[7] was purified by sucrose density gradient centrifugation as described by McMullin and Hallberg[12] with some modifications. An overnight culture (4 ml) of JM109 (pGP57) was used to inoculate 1 liter of Luria–Bertani medium plus ampicillin (50 μg/ml) and grown to OD_{600} = 1.0.

The culture was placed on ice for 15 minutes and then centrifuged for 15 minutes at 6000 RPM in a JA-10 rotor (Beckman Instruments) at 4°C. The cells were resuspended in 40 ml of lysozyme solution (1 mg/ml lysozyme, 30 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 20% sucrose (wt/vol) and were kept on ice for 30 minutes. The resultant spheroplasts were then centrifuged for 10 minutes at 2800g at 4°C. The cells were resuspended in 8 ml of buffer TM (50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$) on ice and sonicated for 5 to 10 seconds. The lysate was then centrifuged at 26,000g for 15 minutes, and the supernatant was collected. Two milliliters (approximately 25 mg total protein) of the supernatant was layered onto a 15% to 30% continuous sucrose gradient made with buffer TM in 25 × 89 mm Beckman Ultra Clear tubes (Palo Alto, CA). An SW28 rotor was run at 25,000 RPM and 4°C for 18 hours. Fractions (2 ml) were collected and analyzed by SDS–PAGE and Coomassie blue staining. Peak fractions containing rHsp60 were used for subsequent experiments (Fig. 1).

Generation of *Salmonella typhimurium* Expressing Chlamydial rHsp60

S. typhimurium strain SL3261[7] was prepared for electrotransformation in water according to BioRad (Richmond, CA) Gene Pulser instructions. Electrocompetent SL3261 cells (1×10^8 colony-forming units in 40 μl) were mixed with pGP57 plasmid DNA (10 μg in 10 μl water) in a 0.2 cm gene pulser cuvette and electrotransformed using a BioRad Gene Pulser (25 μFD and 1.5 kV) equipped with Pulse Controller (1000 OHMS). Ampicillin-resistant colonies were grown to OD_{600} = 0.7 and frozen at −70°C in Luria–Bertani medium supplemented with 10% glycerol. Isolates were assessed by SDS–PAGE for expression of rHSP60. A positive isolate designated SL3261 (pGP57) expressed high levels of rHsp60 (approximately 5% to 10% of bacterial dry weight) and was used for subsequent inoculations of animals (Fig. 2).

Immunization and Assessment of Immune Response

Guinea pigs were immunized subcutaneously (SC) with 50 μg of rHsp60 suspended in phosphate-buf-

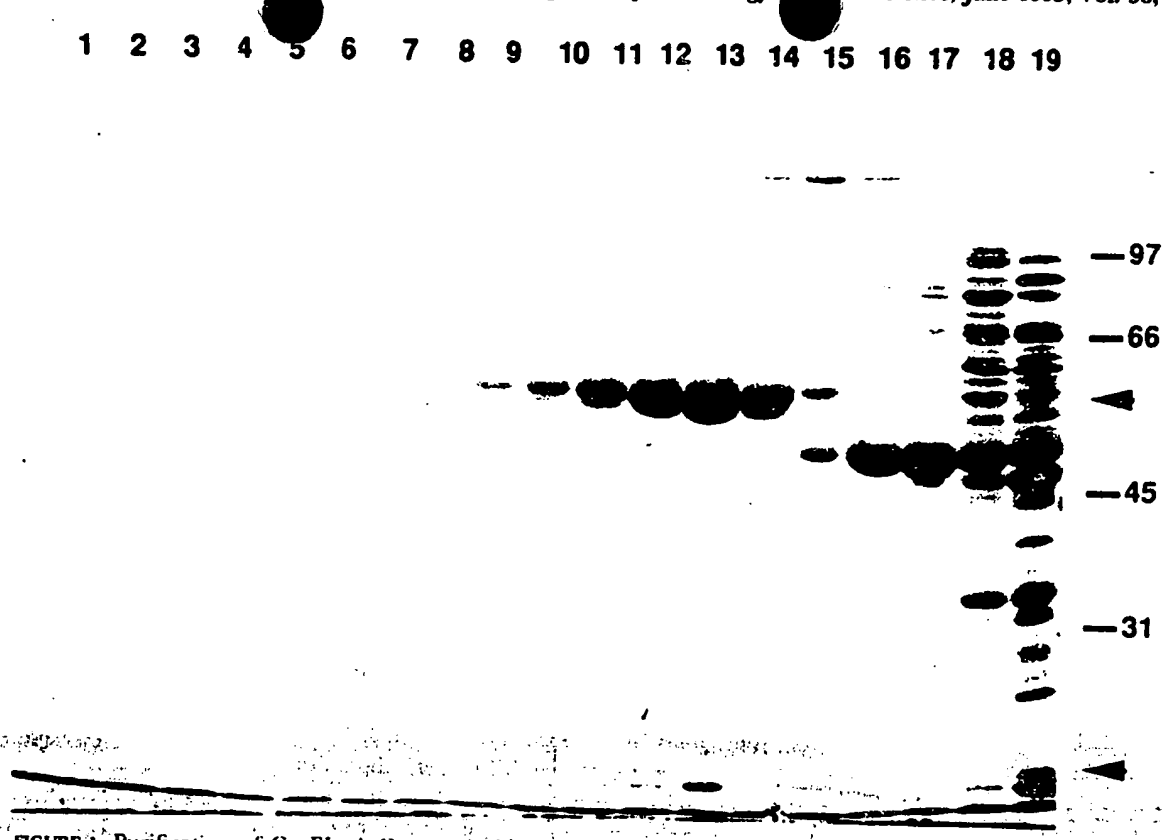

FIGURE 1. Purification of GroEL. A Coomassie blue stained gel of each fraction from the sucrose gradient purification procedure (10 μl per lane). Lane 1 represents the bottom of the gradient, and lane 19 represents the top. Molecular weights (kd) are indicated at the right. The top arrow indicates the position of rHsp60; the bottom arrow indicates the position of rGsp10 (GroES homologue).

fered saline and emulsified 1:1 in Freund's incomplete adjuvant. Immunization with *Salmonella typhimurium aroA* SL 3261 expressing the GPIC Hsp60 was by the instillation of 25 μl of a suspension containing 10^8 viable organisms. Although the amount of rHsp60 delivered by the *Salmonella* cannot be exactly determined, it was probably in the range of 15 to 20 μg.

Serum and ocular secretions were obtained as described previously.[10] Antibodies to Hsp60 were measured using a standard enzyme-linked immunoabsorbent assay with either GPIC elementary bodies or rHsp60 as antigens.[9] For each antigen, 0.5 μg were used per well in a 96-well microtiter plate. Immunoglobulin G (IgG) antibodies to either antigen were measured using peroxidase-labeled rabbit anti-guinea pig IgG (ICN, Costa Mesa, CA), and immunoglobulin A (IgA) antibodies were measured using rabbit anti-guinea pig α-chain (ICN) followed by peroxidase-labeled goat anti-rabbit IgG (ICN).

Assessment of Pathologic Changes

Pathologic changes were assessed using a modification of the 0 to 4+ scale described by Watkins et al.[4] Briefly, palpebral and bulbar conjunctiva are evaluated for erythema, edema, and exudation. The scores are defined as follows: slight erythema or edema of either the palpebral or bulbar conjunctiva, 1+; definite erythema or edema of either the palpebral or bulbar conjunctiva, 2+; definite erythema or edema of both the palpebral or bulbar conjunctiva, 3+; definite erythema or edema of both the palpebral or bulbar conjunctiva and the presence of exudate, 4+. In most experiments, two individuals evaluated the animals although it was not possible to blind the individuals as to the identity of the animals. In the majority of the cases, the scores were reported to be the same by each grader. If there was disagreement, the mean of the scores was taken.

RESULTS

Subcutaneous Immunization With rHsp60

To determine whether immunization with rHsp60 could exacerbate an ocular challenge with viable GPIC, five guinea pigs were injected subcutaneously with 50 μg of purified rHsp60 in Freund's incomplete adjuvant on three separate occasions at 2-week inter-

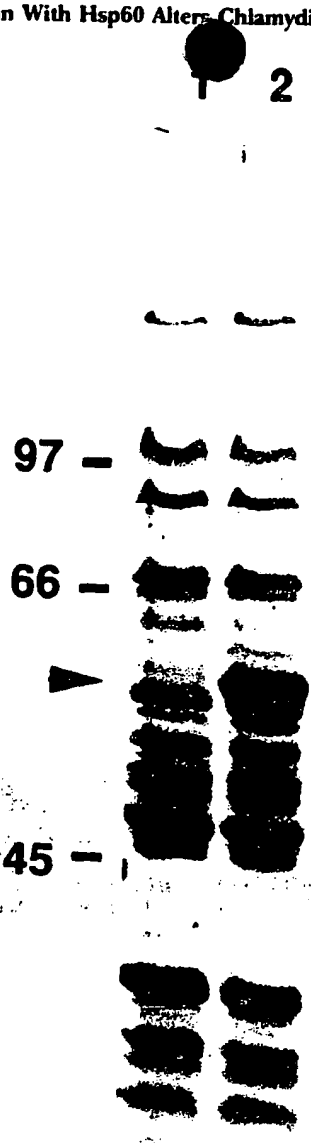

FIGURE 2. *Salmonella typhimurium* SL3261 expressing chlamydial rHsp60. Strains: lane 1, SL3261; lane 2, SL3261(PGP57). Molecular weights (kd) are indicated at the left. The arrow indicates the position of rHsp60.

vals. Two weeks after the last immunization, the immunized animals and four control unimmunized animals were inoculated in the left eye only with 10^6 inclusion-forming units of GPIC, and the development of gross pathology was monitored daily. Conjunctival scrapings were collected every 3 days for the determination of inclusion scores. Interestingly, the animals immunized with rHsp60 did not have enhanced pathology but actually had a small but significantly ($P < 0.0001$) lower level of pathologic changes than did the control unimmunized animals, according to a two-way analysis of variance with repeated measures on two factors (treatment and days) (Fig. 3). Furthermore, it was

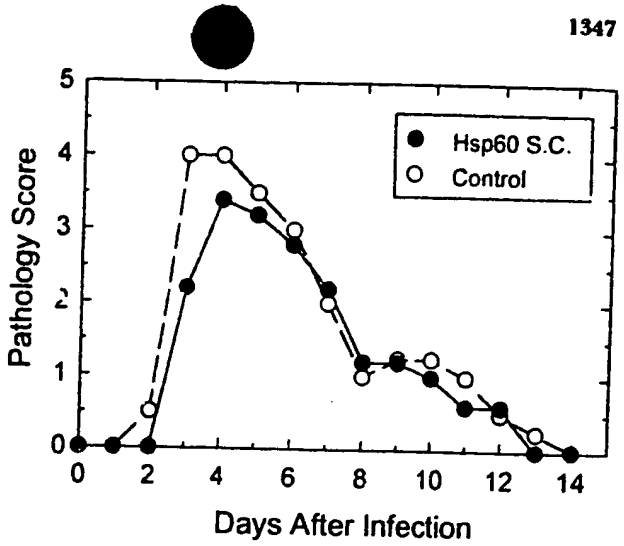

FIGURE 3. Effect of immunization with rHsp60 by the subcutaneous route on the development of pathologic changes in the eye in a primary guinea pig inclusion conjunctivitis infection.

interesting to note that reactions were not seen by 24 to 48 hours after inoculation, which would have been the time a response was expected if there were a strong classic DTH reaction. In contrast, there was no difference in the course of the infection between the two groups as assessed by inclusion scores (Fig. 4).

This experiment was repeated, and a group of animals immunized with UV light-inactivated EB was included to determine if a similar reduced response was observed. Once again, the response of the rHsp60-immunized animals was reduced when compared to the controls, as was the response of guinea pigs immunized with UV–GPIC EB (Fig. 5). As observed in the first experiment, immunization with rHsp60 had no

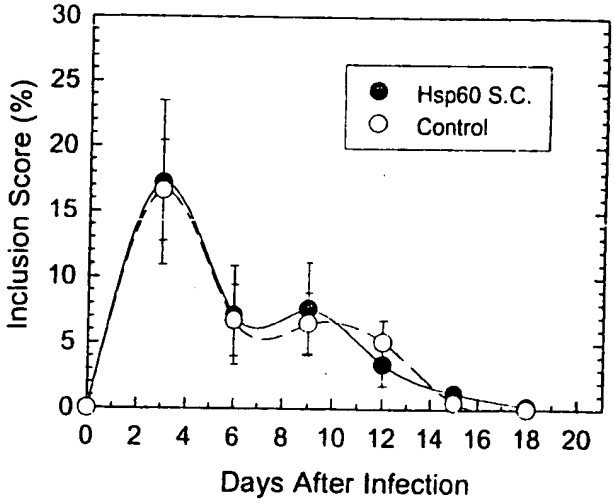

FIGURE 4. Effect of immunization with Hsp60 by the subcutaneous route on the course of ocular infection with guinea pig inclusion conjunctivitis.

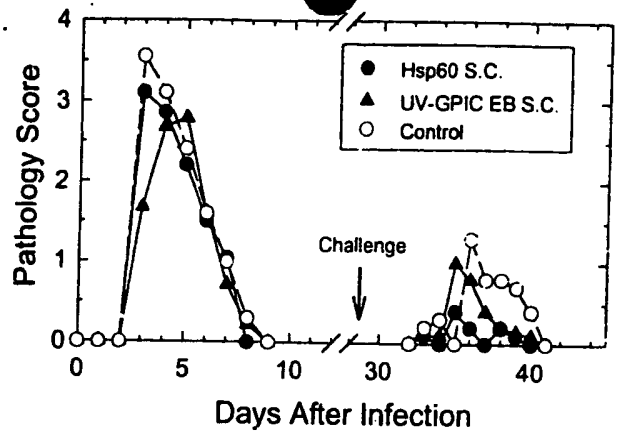

FIGURE 5. Effect of subcutaneous immunization with rHsp60 on a primary and a challenge infection with guinea pig inclusion conjunctivitis (GPIC) in comparison to immunization with ultraviolet light-inactivated GPIC elementary bodies. Animals were given a primary infection only in the left eye. The challenge data in this Figure represent the response in the right eye when reinfected 30 days after infection.

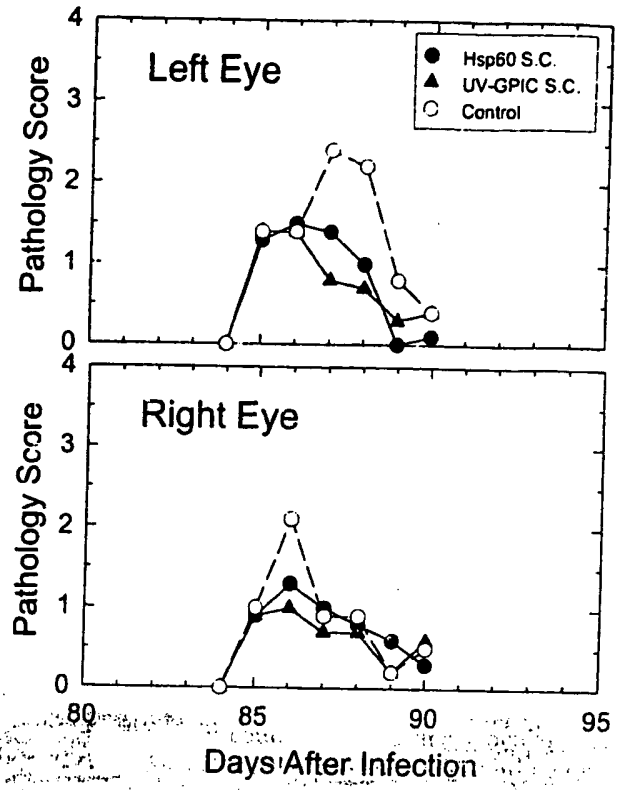

FIGURE 6. Effect of subcutaneous immunization with rHsp60 on challenge infection 80 days after the primary infection. Animals were given a primary infection only in the left eye.

effect on the course of the primary ocular infection when assessed by the percentage of conjunctival cells bearing inclusions on a Giemsa-stained conjunctival scraping, whereas immunization with UV–GPIC did reduce the intensity of infection, as seen before.[15]

The animals were then reinfected with viable GPIC in both eyes either 30 or 75 days after the primary infection to determine if the rHsp60 immunization followed by infection in one eye would accentuate the course of a second infection. In actual clinical disease in humans, it is the repeated antigenic challenge that apparently results in the production of immunopathology, so this experiment was more realistic in this regard. Reinfection with live GPIC in the previously infected left eye did not result in any obvious pathologic changes (data not shown). In contrast to the left eye, pathologic changes were noted in the previously uninfected right eye after the infection on day 30 (Fig. 5). However, the pathologic response was significantly lower ($P < 0.001$) in the immunized group when compared to UV–GPIC-immunized and unimmunized controls.

When separate immunized groups, also given a primary infection in the left eye, were reinfected in both eyes 75 days after the primary infection, pathologic changes were seen in both eyes; in both scenarios, the pathologic response was lower in the UV–GPIC-immunized and the rHsp60-immunized groups than in the control group (Fig. 6). These data were provocative because they were contrary to what was expected. Rather than producing an exacerbated response by immunizing with rHsp60 before ocular challenge with viable chlamydiae, immunization actually seemed to provide a statistically significant measure of protection against pathologic changes, albeit not great in magnitude.

The IgG and IgA antibody responses to Hsp60 in ocular secretions before reinfection at 30 and 75 days after the primary infection was determined to assess the degree of reactivity to each antigen. At each time, the response to Hsp60 was significantly greater in the rHsp60-immunized guinea pigs when compared to controls, although a strong response was also noted in the group immunized with UV–GPIC EB (Table 1).

Immunization With Attenuated *Salmonella typhimurium* Expressing rHsp60

One explanation for these results was that SC immunization was unable to elicit T cells with the appropriate receptors necessary to home to the mucosal site. Thus, it may be essential to stimulate a local mucosal T cell response for a local DTH response to be elicited by GPIC challenge. To test this hypothesis, guinea pigs were immunized with attenuated *S. typhimurium* expressing rHsp60. One group of guinea pigs was immunized SC with 50 μg of purified rHsp60 in Freund's incomplete adjuvant, followed 2 weeks later with ocular inoculation of recombinant bacteria, and 2 weeks

TABLE 1. Antibody Titers to Hsp60 in Ocular Secretions Before Reinfection at Either 30 or 75 Days After a Primary Ocular Infection

	Day 30		Day 75	
Immunization	*Immunoglobulin G*	*Immunoglobulin A*	*Immunoglobulin G*	*Immunoglobulin A*
Hsp60	3.16 ± 0.39*	3.22 ± 0.34	2.44 ± 0.25	2.26 ± 0.13
UV–GPIC	2.26 ± 0.49	1.90 ± 0.37	1.04 ± 0.61	1.36 ± 0.25
None	1.72 ± 0.46	1.90 ± 0.37	0.90 ± 0.88	0.73 ± 0.85

* Log_{10} ± SD.

after that with a combined ocular bacterial inoculation and SC purified rHsp60 inoculation. A second group received three SC inoculations with rHsp60 in Freund's incomplete adjuvant at 2-week intervals. A third group was not immunized. Both immunization regimens resulted in the production of specific serum and secretion IgG (Table 2) when assessed just before the primary infection, indicating that the system was indeed primed. The IgG response in the SC immunized group was much greater than the response elicited by the combined ocular and SC immunization regimen.

Two weeks after the last immunization, all guinea pigs were given a primary infection with 10^6 inclusion-forming units of GPIC in both eyes. As before, the pathologic response resulting from the primary infection was lower in the SC immunized group than in the control group (data not shown). The combined SC–ocular immunized group had an equally low response. When the course of the infection was compared between the groups, no differences in the level or length of infection were noted, and the infection was, in fact, similar to the infection seen in Figure 4.

However, on reinfection 30 days after the primary infection, only the SC group demonstrated a decreased pathologic reaction. The SC–ocular combined immunization group had the same level of response as did the control animals (Fig. 7A). Once again, responses were not seen at 24 to 48 hours after inoculation. Similar data were obtained when the experiment was repeated (Fig. 7B). When the IgG antibody response to Hsp60 was determined in ocular secretions before the day 30 challenge, SC immunization was once again found to elicit a higher response than the SC–ocular immunization group (3.38 ± 0.22 versus 2.28 ± 0.27, respectively; $P < 0.0001$).

TABLE 2. Antibody Titers to Hsp60 in Serum and Ocular Secretions Before the Primary Infection

Immunization	*Serum IgG*	*Ocular Secretions IgG*
Subcutaneous ocular	3.70 ± 0.32*	0.70 ± 0.11
Subcutaneous alone	3.82 ± 0.22	1.96 ± 0.64

* Log_{10} ± SD

DISCUSSION

In this study, we attempted to determine the effect of immunization of guinea pigs with purified rHsp60 on subsequent ocular infection with GPIC. Because Hsp60 has been shown to elicit a DTH response in

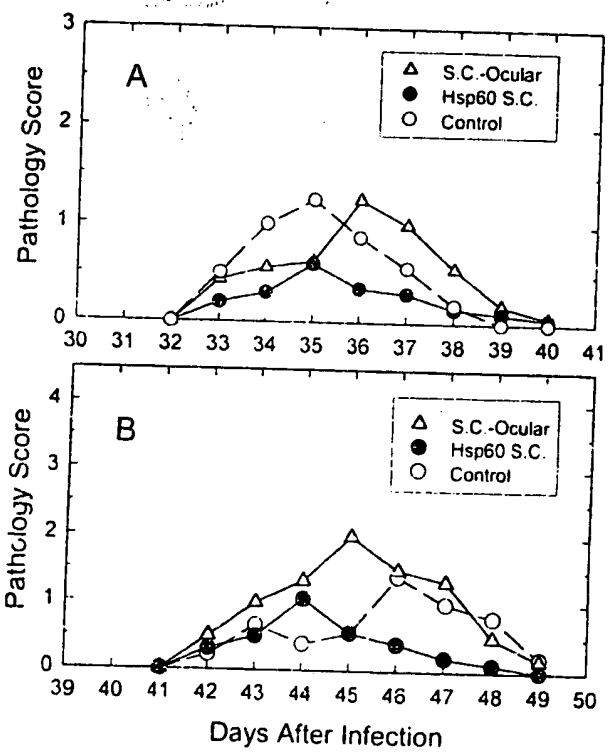

FIGURE 7. Comparison of subcutaneous immunization with rHsp60 and combined subcutaneous immunization with rHsp60 and ocular immunization with viable *Salmonella typhimurium* expressing guinea pig inclusion conjunctivitis rHsp60. Panels A and B represent two separate experiments.

previously infected guinea pigs,[16] it was anticipated that immunization with rHsp60 would elicit an exacerbated response when guinea pigs were infected in the eye with viable GPIC. However, an exacerbated reaction in the conjunctiva was not noted in any of the experiments, and no reactions were noted at 24 to 48 hours after inoculation, which is the time traditionally associated with the development of a DTH reaction.

Of significance was the observation that immunization with purified rHsp60 by the subcutaneous route actually reduced the pathologic response in the conjunctiva after a primary infection when compared to unimmunized controls. Furthermore, when the animals were reinfected, a "protective" effect was still noted even though both groups had the same experience with viable GPIC. This occurred when animals were reinfected soon after recovery from the primary infection or even when they were reinfected approximately 2 months later. It is interesting to note that if guinea pigs were reinfected shortly after infection in the eye initially infected, no pathologic response was seen in any group. It has been observed that immunity to reinfection at this time after a primary infection is high,[14] and it is possible that sufficient antibody and T cells were already present in the eye after the primary infection to eliminate the organism quickly. We have demonstrated in the GPIC–guinea pig genital tract model that complete immunity to reinfection is dependent on the number of specific T cells at the local site.[15] Immunization with whole GPIC elementary bodies did not reduce the pathologic response to reinfection.

The mechanism of this protective response remains to be defined, but it could be related to a strong antibody response to Hsp60 in ocular secretions. The subcutaneous immunization regimen elicited high titers of IgG and IgA antibodies to Hsp60. It is conceivable that the antibody could "block" the Hsp60 or effect its removal so that a DTH response is not initiated or is diminished. Although it is also possible that the DTH response was not primed by immunization through the SC route, previous studies have demonstrated that immunization of guinea pigs with purified Hsp60 by the SC route does elicit strong lymphocyte proliferative responses when peripheral blood lymphocytes are incubated with Hsp60 antigen in vitro (Kincy and Rank, unpublished data, 1992). We have noted[16] in a murine model of reactive arthritis that in the absence of antibody, chlamydial antigen remains at the local site longer, and the pathologic reaction is markedly enhanced. Thus, the arthritis model indicates that antibody can downregulate chlamydial disease.

In contrast, when animals were immunized with a regimen that combined subcutaneous immunization with rHsp60 and immunization locally in the conjunctiva with avirulent *S. typhimurium* expressing GPIC rHsp60, no protective response was noted. In fact, the intensity of the pathologic response was similar to that of the control unimmunized animals. These data suggest that the route of exposure to Hsp60 may be critical in the development of a pathologic response, although further experiments with immunization by other mucosal routes will be necessary to confirm these observations. In natural exposure of individuals to *C. trachomatis* in trachoma-endemic areas, people are exposed by the ocular route, and a DTH response occurs even in situations in which apparently few organisms can be isolated.[3] Similarly, in the guinea pig, immunization by the ocular route with rHsp60, expressed by a viable organism that can gain access to the intracellular environment, also elicits a response comparable to the natural infection. However, if animals are previously immunized by the same rHsp60 through a parenteral route, the pathologic response on challenge with viable organisms is diminished. Thus, in the development of a vaccine for trachoma or for genital tract disease, immunization through the mucosal route may actually be less desirable because of the potential for the induction of a pathologic event, especially because it is not clear if Hsp60 is the only antigen capable of eliciting an immunopathologic response.

An explanation for this phenomenon might be that previous local immunization followed by GPIC infection may elicit a larger population of Hsp60-specific T cells that could home to the local site, thereby shifting the balance away from any blocking function of local antibody. This explanation is more likely for the results of the secondary infection because that response is predominantly a DTH response.[5] An alternative explanation is that the immunization dose associated with the *S. typhimurium* recombinant may have been insufficient to elicit the "protective" response in comparison to immunization with purified RHsp60 administered subcutaneously. However, we have noted that ocular immunization with the *S. typhimurium* recombinant is sufficient to elicit a local mucosal antibody response to *Salmonella* antigens (data not shown).

In contrast to reinfection, the primary GPIC infection in the guinea pig results in a vigorous acute inflammatory reaction that can mask a DTH response. It is not clear how immunization with rHsp60 could alter this response because acute inflammation arises from mechanisms not necessarily related to the acquired immune response.

Key Words

Chlamydia, heat shock protein, trachoma, guinea pig, immunization

Acknowledgments

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References

1. Grayston JT, Wang S-P, Yeh LJ, Kuo CC. Importance of reinfection in the pathogenesis of trachoma. *Rev Infect Dis.* 1985;7:717–725.
2. Grayston JT, Wang S-P. New knowledge of chlamydiae and the diseases they cause. *J Infect Dis.* 1975; 132:87–105.
3. Taylor HR, Johnson SL, Prendergast RA, Schachter J, Dawson CR, Silverstein AM. An animal model of trachoma: II: The importance of repeated reinfection. *Invest Ophthalmol Vis Sci.* 1982;23:507–515.
4. Watkins NG, Hadlow WJ, Moos AB, Caldwell HD. Ocular delayed hypersensitivity: A pathogenetic mechanism of chlamydial conjunctivitis in guinea pigs. *Proc Natl Acad Sci USA.* 1986;83:7480–7484.
5. Monnickendam MA, Darougar S, Treharne JD, Tilbury AM. Development of chronic conjunctivitis with scarring and pannus, resembling trachoma, in guinea pigs. *Br J Ophthalmol.* 1980;64:284–290.
6. Morrison RP, Belland RJ, Lyng K, Caldwell HD. Chlamydial disease pathogenesis: The 57-kD chlamydial hypersensitivity antigen is a stress response protein. *J Exp Med.* 1989;170:1271–1283.
7. Morrison RP, Su H, Lyng K, Yuan Y. The *Chlamydia trachomatis hyp* operon is homologous to the *groE* stress response operon of *Escherichia coli*. *Infect Immunol.* 1990;58:2701–2705.
8. Taylor HR, Johnson SL, Schachter J, Caldwell HD, Prednergast RA. Pathogenesis of trachoma: The stimulus for inflammation. *J Immunol.* 1987;138:3023–3027.
9. Rank RG, Batteiger BE, Soderberg LSF. Susceptibility to reinfection after a primary chlamydial genital infection. *Infect Immunol.* 1988;56:2243–2249.
10. Rank RG, Barron AL. Specific effect of estradiol on the genital mucosal antibody response in chlamydial ocular and genital infections. *Infect Immunol.* 1987; 55:2317–2319.
11. Schachter J. Chlamydiae. In: Lennette EH, ed. *Manual of Clinical Microbiology.* Washington, DC: American Society for Microbiology; 1980:357–364.
12. McMullin TW, Hallberg RL. A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by *Escherichia coli* groEL gene. *Mol Cell Biol.* 1988;8:371–380.
13. Rank RG, Batteiger BE, Soderberg LSF. Immunization against chlamydial genital infection in guinea pigs with UV-inactivated and viable chlamydiae administered by different routes. *Infect Immunol.* 1990; 58:2599–2605.
14. Ahmad A, Dawson CR, Yoneda C, Togni B, Schachter J. Resistance to reinfection with a chlamydial agent (guinea pig inclusion conjunctivitis). *Invest Ophthalmol Vis Sci.* 1977;16:549–553.
15. Igietseme JU, Rank RG. Susceptibility to reinfection after a primary chlamydial genital infection is associated with a decrease of antigen-specific T cells in the genital tract. *Infect Immunol.* 1991;59:1346–1351.
16. Rank RG, Ramsey KH, Hough AJ Jr. Antibody-mediated modulation of arthritis induced by Chlamydia. *Am J Pathol.* 1988;132:372–381.

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From: Portner, Ginny
Sent: Wednesday, August 02, 2000 4:39 PM
To: STIC-ILL
Subject: 09/452,287 Chlamydia

Polio virus-derived epitope expression vectors- Sabin-1 vector construction, live attenuated oral recombinant vaccine safety and efficacy; potential human papilloma virus, Chlamydia trachomatis, HIV virus, SIV virus cloning (conference abstract)
AUTHOR: Evans D J; Minor P D; Almond J W
CORPORATE SOURCE: Department of Microbiology, University of Reading, London Road, Reading, UK.
JOURNAL: **J.Appl.Bacteriol. (69, 6, xiii) 1990**
CODEN: JABAA4
LANGUAGE: English

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task is the production of peptides in which the secondary structure is constrained so as to better represent the native protein.

Humoral immune responses require the co-operation of both B-cells and helper T-cells. Consequently a synthetic immunogen must include epitopes appropriate for recognition by both of these elements of the immune system.

Finally, small peptides are usually poorly immunogenic and therefore methods for improving the levels of response are important. Examples of how this may be achieved will be given.

Poliovirus-derived Epitope Expression Vectors. By D.J. EVANS, P.D. MINOR* and J.W. ALMOND (*Department of Microbiology, University of Reading, London Road, Reading and *National Institute for Biological Standards and Control, South Mimms, Herts, UK*).

As part of studies to improve the safety and efficacy of the live attenuated oral poliovirus vaccines currently in use, we have used the Sabin 1 strain as the basis for the construction of intertypic antigen chimaeras. Antigenic determinants from the less stable type 2 and type 3 strains have been engineered onto the surface of the multiply attenuated type 1 strain, via the mutagenesis of an infectious cDNA clone of Sabin 1. Our studies have initially concentrated upon antigenic site 1, a linear epitope occupying residues 92–103 of capsid protein VP1, which projects at the pentameric apex of the virus particle. Viable chimaeric virus particles, in which this region of Sabin 1 has been replaced with the corresponding sequence from a type 3 strain, have antigenic and immunogenic characteristics of both the type 1 'backbone' and the inserted sequence. To allow more extensive modification of antigenic site 1 of Sabin 1 we have constructed a series of mutagenesis cassette vectors. Unique restriction sites have been introduced flanking the region of the cDNA encoding antigenic site 1, thereby facilitating the rapid replacement of the region with complementary oligonucleotides encoding sequences of choice. The cassette vectors have been used to introduce sequence encoding known or predicted antigenic sites, from a range of pathogens including human and simian immunodeficiency viruses, human papillomavirus type 16 and *Chlamydia trachomatis*.

Expression and Secretion of Repertoires of VH Domains in *Escherichia coli*: Isolation of Antigen Binding Activities. By A. GRIFFITHS, E. SALLY WARD, D.H. GÜSSOW, P.T. JONES and G. WINTER (*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*).

The Fv fragment of an antibody forms the antigen-binding site, and consists of heavy (VH) and light chain (VL) variable domains packed together by hydrophobic interactions. Each of these variable domains has three hypervariable loops (complementarity determining regions; CDRs), supported on a framework of beta-sheets, and most of the antibody contacts with antigen are made by these hypervariable residues. We are interested in the determinants of antibody affinity and specificity, and are using an *Escherichia coli* secretion vector to clone and express antibody variable domains which have been manipulated *in vitro*. Leakage of expressed protein from the periplasm into the culture supernatant fluid allows the use of ELISAs to analyse supernatant fluids directly for antigen binding. We have expressed the anti-lysozyme D1.3 Fv in this system, and found that for this antibody, VH binds antigen with high affinity in the absence of VL. This led us to generate repertoires of VH domains for the expression of binding activities in *E. coli*. To produce these repertoires, rearranged VH genes have been cloned from antibody-producing cells of immunized mice using the polymerase chain reaction. VH domains with desired binding activities have been identified by screening with ELISA, and several of these VH domains which bind to hen egg lysozyme have been purified and characterized kinetically. Current work is directed towards the matching of VH with VL domains, and Fd fragments with light chains, to generate Fv and Fab fragments respectively.

The Use of Monoclonal Antibodies in Sexually Transmitted Infections. By CATHERINE ISON (*St. Mary's Hospital Medical School, Paddington, London W2 1PG, UK*).

Monoclonal antibodies have been used in sexually transmitted infections in four main areas. The detection of antigen in clinical samples, the identification and epidemiology of organisms isolated from patients and to aid studies on the pathogenesis or aetiology of particular infections.